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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | |
|---|---|---|
| Appln. Serial No.: | 09/373,272 | Group Art Unit: 1635 |
| Filing Date: | 08/12/1999 | Examiner: Epps, Janet |
| Applicant(s): | Austin-Phillips et al. | Attorney Docket No.: 09820.114 |

Title: Transgenic Plants as an Alternative Source of Lignocellulosic-Degrading Enzymes

## DECLARATION UNDER RULE 131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

To the Commissioner:

Your Declarants, Sandra Austin-Phillips, Richard R. Burgess, Thomas L. German, and Thomas Ziegelhoffer, do hereby declare and state as follows:

1. We are each co-inventors of Claims 27, 28, and 31-40 of the above-identified patent application and co-inventors of the subject matter described and claimed therein.

2. The above-identified application for patent was filed on August 12, 1999, and is a continuation-in-part of parent application Serial No. 08/883,495, filed June 26, 1997 (and issued as U.S. Patent No. 5,981,835). A true and complete copy of the parent application as filed (S.N. 08/883,495) is attached hereto as Exhibit A, and is incorporated herein.

3. We collectively submit that the subject matter of Claims 27, 28, and 31-40 of the above-identified patent application are enabled by the parent application attached hereto as Exhibit A. Specifically, please see pages 6 and 7 and Figs. 1-5 of Exhibit A. Fig. 1 of Exhibit A is a schematic diagram of an gene construct to express the E2 cellulase of *T. fusca* in plants. Similarly, Fig. 2 of Exhibit A is a schematic diagram of a gene construct to express the E3 cellulase of *T. fusca* in plants. Fig. 3 of Exhibit A is a western blot evidencing that the present inventors had expressed *T. fusca* E2 cellulase in tobacco. Fig. 4 of Exhibit A is a western blot evidencing that the present inventors had expressed *T. fusca* E3 cellulase in tobacco. Fig. 5 of Exhibit A is a western blot evidencing that the present inventors had expressed *T. fusca* E2 cellulase in alfalfa. See also Examples 1, 2, and 3 of Exhibit A, at page 22, line 20, to page 25, line 26. These examples describe the expression of *T. fusca* cellulases E2 and E3 in alfalfa and in tobacco. Lastly, see Example 4, beginning at page 26, line 1 of Exhibit A, which describes using the transgenic plants in silage. Thus, we respectfully submit that all of present Claims 27, 28, and 31-40 are entitled to priority to the June 26, 1997 filing date of the parent application.

4. Prior to September 12, 1996, we had conceived of and reduced to practice the invention described and claimed in the present application as evidenced by Exhibits B and C, attached hereto and incorporated herein. All of the dates redacted from Exhibits B and C are prior to September 12, 1996.

5. Attached hereto as Exhibit B is an excerpt from my, Thomas Ziegelhoffer's, laboratory notebooks. I personally signed and dated the notebook pages contained in Exhibit B, contemporaneously with the performance of the experiments detailed in Exhibit B. All of the dates redacted from Exhibit B are prior to September 12, 1996. I, Thomas Ziegelhoffer, declare that the lab

notebook pages shown in Exhibit B detail the construction of expression vectors containing the E2 and E3 genes from *T. fusca*. The first seven (7) pages of Exhibit B document my successful efforts to clone the E2 and E3 cellulase genes of *T. fusca* and to insert the cloned genes into a suitable construct for transforming a plant host. Specifically, at the first page of Exhibit B, I, Thomas Ziegelhoffer, titled the experiment "Expression of cellulases in plants...." I then recorded the rational for the subsequent experiments as follows: "For an initial set of constructs, fuse E2 and E3 genes of Thermospora fusca to MAC promoter and NOS terminator in PCGN1578 constructs...." Sheets 6-13 of Exhibit B show that tobacco plants transformed with the vectors described earlier in Exhibit B did express the E2 and E3 genes as shown by Western blot.

6. Attached hereto as Exhibit C is true copy of a cover letter authored by Cheryl E. Gest of the University of Wisconsin and an attached grant application entitled "Transgenic Alfalfa as an Alternative Economical Source of Lignocellulosic Degrading Enzyme for Use in Biomass Conversion." Exhibit C is incorporated herein. We, the Declarants Sandra Austin-Phillips, Richard R. Burgess, and Thomas L. German, authored the grant proposal presented in Exhibit C. In Exhibit C, page 1, Section 2, paragraph A, it is noted that the goal of the research is to construct plant expression vectors coding for cellulases. Further, Exhibit C indicates that an alternative source for cloned *T. fusca* E2 and E3 had been secured (courtesy of Dr. P. Wilson at Cornell University). Also note that paragraphs 2A *et seq.* of Exhibit C detail a strategy for constructing the expression vectors that are currently claimed in the present application.

7. We, Sandra Austin-Phillips, Richard R. Burgess, Thomas L. German, and Thomas Ziegelhoffer (individually and collectively), do hereby declare that all statements made herein of our own knowledge (individual and collective) are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 1/20/04 — Sandra Austin-Phillips
**Sandra Austin-Phillips**

Date: _____ — _____
**Richard R. Burgess**

Date: 1/20/04 — _____
**Thomas L. German**

Date: 1/20/04 — _____
**Thomas Ziegelhoffer**

7. We, Sandra Austin-Phillips, Richard R. Burgess, Thomas L. German, and Thomas Ziegelhoffer (individually and collectively), do hereby declare that all statements made herein of our own knowledge (individual and collective) are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____

**Sandra Austin-Phillips**

Date: 1/23/04

Richard R Burgess

**Richard R. Burgess**

Date: _____

**Thomas L. German**

Date: _____

**Thomas Ziegelhoffer**

# TRANSGENIC PLANTS AS AN ALTERNATIVE SOURCE OF LIGNOCELLULOSIC-DEGRADING ENZYMES

SANDRA AUSTIN-PHILLIPS
RICHARD R. BURGESS
THOMAS L. GERMAN
THOMAS ZIEGELHOFFER

# TRANSGENIC PLANTS AS AN ALTERNATIVE SOURCE OF LIGNOCELLULOSIC-DEGRADING ENZYMES

SANDRA AUSTIN-PHILLIPS
RICHARD R. BURGESS
THOMAS L. GERMAN
THOMAS ZIEGELHOFFER

Priority is claimed to provisional application serial number 60/028,718, filed October 17, 1996.

## FIELD OF THE INVENTION

The present invention is directed to the production of cellulose-degrading enzymes in genetically recombinant plants and the recombinant plants themselves.

## BIBLIOGRAPHY

Complete bibliographic citations for the non-patent references discussed hereinbelow are included in the Bibliography section, immediately preceding the claims. All of the references cited below are incorporated herein by reference.

## DESCRIPTION OF THE PRIOR ART

Lignocellulosic plant matter, such as agricultural and forestry waste, as well as energy crops produced specifically for biomass, offer tremendous potential for the renewable production of fuel and as chemical feedstocks. However, production cost for desired products such as alcohols from lignocellulosic material is significantly higher than the production cost of equivalent alternatives. However, the prospect, either real or perceived, of limited fossil fuel reserves, along with the geo-political issues which swirl

about petroleum-producing countries and regions, renders the production of basic chemical feedstocks and fuels from local, renewable sources an attractive alternative to fossil fuels.

For instance, alcohols have the potential to be excellent alternative transportation fuels if their production costs can be lowered. Brazil has sponsored several programs to replace car engines which run on gasoline alone to engines which run on ethanol or a gasoline-ethanol mix.

Unfortunately, the production of ethanol and other feedstock chemicals from lignocellulosic material is far more complex than an analogous production utilizing a starch-based starting material. Compared to lignocellulosic materials, starch is a simple polymer which is readily hydrolyzed to glucose. Yeasts can then be used to convert the glucose to ethanol.

In contrast, lignocellulosic biomass is a much more complex substrate in which crystalline cellulose is embedded within a matrix of hemicellulose and lignin. The intricate structure and relative inaccessibility of these substrates requires pre-treatment for the disruption of the lignocellulosic material, as well as hydrolysis of hemicellulose and lignin into xylose and phenolic compounds, respectively. (See, for instance, *Micelli et al.* (1996), *Belkacemi et al.* (1996), and *Grohmann et al.* (1992).)

Several enzymes which degrade lignocellulosic material, commonly referred to as "cellulases," are known. The term "cellulase" shall be used herein to refer to any and all enzymes which catalyze the cleavage of cellulosic or lignocellulosic materials. Explicitly, but not exclusively, included within this definition are those cellulases which fall under the Enzyme Classification heading EC 3.2.1.x. Various genes encoding cellulases have also been isolated and characterized.

For instance, genes which encode endoglucanases from the fungus *Trichoderma reesei* are known and have been successfully incorporated and expressed in yeast. See, for instance, *Pentilla et al.* (1987). Likewise, cellulase E2 (EC 3.2.1.4) and cellulase E3 (EC 3.2.1.91) from the thermo-tolerant bacterium *Thermomonospora fusca* are known. See *Lao et al.* (1991), *Spezio et al.* (1993) and *Zhang et al.* (1995).

From a functional viewpoint, cellulases are catagorized into two large sub-groups based upon whether they catalyze cleavage from the cellulose chain ends (exocellulases) or if they catalyze cleavage in the middle of the cellulose chain (endocellulases). For instance, cellobiohydrolase I of *T. reesei* (CBH I, EC 3.2.1.91) is an exocellulase, which degrades crystalline cellulose by cleavage from the chain ends. By way of further illustration, CBH I is a 68 kDa protein with a two-domain architecture which is shared by many cellulases. In this chemical architecture, a large catalytic domain is joined to a cellulose-binding domain (CBD) through a flexible linker region. See *Divne et al.* (1994). Similarly, cellulase E3 of *T. fusca* is also an exocellulase.

Different types of cellulases exhibit synergistic activity on complex substrates. This synergism, especially between exocellulases, is believed to be due to differences in their patterns of absorption to and hydrolysis of complex cellulose substrates. See *Henrissat et al.* (1995).

Illustratively, cellulase E2 of *T. fusca* is a 40 kDa endocellulase which cleaves the cellulose chain internally. Such cleavage generates more chain ends for attack by exocellulases. Consequently when CBH I, E2, and E3 cellulases are combined, their activity together is approximately 5-fold greater than their additive individual activities. (See, for instance, *Irwin et al.* (1993) and WO 94/26880.) It is important to note that proteolytic fragments of cellulases can substitute for the intact enzymes in synergistic mixtures. For example, when combined with *T. fusca* E3 and CBH I, the catalytic domain of *T. fusca* E2 ("E2cd") is as active as the intact enzyme in the digestion of filter paper substrate, *Irwin et al.* (1993).

A wide range of compositions containing cellulases are described in the patent literature. For instance, *Evans et al.*, U.S. Patent No. 5,432,074, describe the use of a formulation consisting essentially of a combination of xylanase and xylosidase, but being essentially free of glucanase and cellobiohydrolase. The formulation also contains a lactic acid-producing bacteria. The formulation is used to treat silage to increase its nutritive value. In operation, the action of the xylanase and xylosidase enzymes degrades

non-cellulosic polysaccharides found in the silage material thereby producing sugars for fermentation.

Heterodimers of different types of cellulose-degrading enzymes are described in WO 94/29460. Here, a β-glucosidase molecule and a cellobiohydrolase molecule (*i.e.*, an exocellulase) are chemically bonded to one another by a crosslinking reagent to yield a single molecule which retains the enzymatic activities of the two separate molecules.

Expression constructs which contain cellulase genes for the transformation of yeast have been constructed. For example, *Knowles et al.*, U.S. Patent No. 5,529,919, describe the transformation of *S. cerevisiae* to contain and express a thermostable β-endoglucanase (EG I) of *T. reesei*.

Likewise, attempts have been made to produce transgenic plants which express cellulose-degrading enzymes. *Aspegren et al.* (1995) describe transgenic suspension-cultured barley cells which express EG I of *T. reesei*. The cells were transformed by particle bombardment and transformed cells selected by a co-transformed antibiotic resistance marker. However, no attempt was made to regenerate complete plants from the cultured cells. Of particular note, this reference states that the production of β-glucanases in plant cells may be hampered by the fact that these enzymes catalyze the hydrolysis of essential cell wall components. Attempts by these authors to stably transform tobacco cells with the same construct used to successfully transform the suspended barley cells failed. Here, the authors observed that after transient expression in tobacco protoplasts, cell wall synthesis never resumed.

## SUMMARY OF THE INVENTION

The present invention is drawn to genetically recombinant plants which contain one or more exogenous gene sequences which encode one or more cellulose-degrading gene products. The gene product or products are expressed in recoverable quantities in the recombinant plants and can be isolated from the plants, if desired. In the preferred embodiment, the genetically recombinant plant expresses the gene product constituitively.

However, the invention also encompasses recombinant plants which express the gene product stage-specifically or tissue-specifically. For example, the gene product or products can be expressed in a plant tissue such as the seeds, fruit, leaves, or tubers of the transformed plant host.

The invention is further drawn to recombinant plants as noted above, wherein the plant contains two exogenous genes whose respective gene products are expressed independently of one another. This allows for different types of cellulases to be expressed in different locations within the same recombinant plant. For example, the plant host can be transformed to express two or more heterologous cellulases in different sub-cellular compartments such as the plastid, cytosol, endoplasmic reticulum, mitochondrion, inclusion body, or vacuole. In addition, chloroplast targeting can also be accomplished through the use of direct chloroplast transformation, an approach that circumvents many of the problems associated with expression of heterologous genes in the nuclear genome. *Carrer et al.* (1993), *McBride et al.* (1994).

The invention is further drawn to a method for producing cellulose-degrading enzymes. The method comprises transforming a plant host with one or more exogenous genes which encode one or more cellulose-degrading gene products such that the gene product or products are expressed in recoverable quantities. The plant matter containing the expressed protein can be used directly as a feedstock for biomass conversion, or, if desired, the exogenous enzymes so produced can be isolated and purified.

The cellulases produced by the transgenic plants of the present invention can be utilized in the same manner as conventionally-derived cellulases. For instance, cellulases produced by the transgenic plants of the present invention can be isolated and used in fermentation processes such as brewing and wine-making. Here, the cellulases function to hydrolyze cellulose and $\beta$-glucans during fermentation. Or, as described in Example 4, below, whole plants transformed to express cellulases can be used directly or added to ensiled plant matter to increase the extent of fermentation of the ensiled matter. Plants transformed to express functional cellulases may also be fed directly to livestock, where the cellulase activity aids in the digestion of lignocellulosic substrates.

Cellulases produced in the transgenic plants of the present invention can also be utilized in the production of ethanol and other feedstock chemicals from lignocellulosic substrates.

Cellulases produced by transgenic plants of the present invention can also be used in the textile, pulping, and paper-making industries. For instance, cellulases are conventionally used to treat denim fabrics to give them a "stone-washed" appearance. Cellulases are also used to modify paper pulps by digesting the cellulose fibers contained within the pulp. The cellulases produced by the transgenic plants described herein can be used in this fashion.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Fig. 1** is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E2 of *T. fusca*. Promoters and structural genes are depicted as arrows which indicate the direction of transcription. Terminators are depicted as boxes. NPT II = neomycin phosphotransferase; Met-E2m = *T. fusca* E2 (mature form with N-terminal methionine added); MAS-ter = mannopine synthetase terminator; TML-ter = tumor morphology left terminator; MAC-pro = hybrid "MAC" promoter.

**Fig. 2** is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E3 of *T. fusca*. Promoters and structural genes are depicted as arrows which indicate the direction of transcription. Terminators are depicted as boxes. NPT II = neomycin phosphotransferase; Met-E3m = *T. fusca* E3 (mature form with N-terminal methionine added); MAS-ter = mannopine synthetase terminator; TML-ter = tumor morphology left terminator; MAC-pro = hybrid "MAC" promoter.

**Fig. 3** is a western blot analysis evidencing the expression of *T. fusca* E2 cellulase in tobacco transformed to contain the expression construct depicted in Fig. 1.

Fig. 4 is a western blot analysis evidencing the expression of *T. fusca* E3 cellulase in tobacco transformed to contain the expression construct depicted in Fig. 2.

Fig. 5 is a zymogram gel assay evidencing the expression of active *T. fusca* E2 cellulase in alfalfa transformed to contain the expression construct depicted in Fig. 1.

## DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to genetically recombinant plants which express one or more exogenous cellulose-degrading (cellulase) enzymes. The invention is further drawn to a method of producing cellulases in plants. The invention allows the production of cellulases using the means and methods of large-scale agriculture rather than the conventional route of large-scale fermentation of the bacteria or fungi which are native producers of the cellulases.

The recombinant plants are produced by incorporating into a plant host genome one or more expression constructs comprising a DNA sequence which encodes a protein having cellulose-degrading activity. Introduction of the exogenous gene or genes into the plant is accomplished by any means known to the art. The expression constructs described hereinbelow enable the stable transformation of plants with one or more genes which encode cellulose-degrading enzymes. The constructs include a DNA coding sequence which encodes a cellulase (as that term is described herein) which is operatively linked to regulatory sequences which direct constituitive, stage-specific, or tissue-specific expression of the cellulase DNA.

### Cellulose-Degrading Enzymes (Cellulases) and Genes:

As noted above, the term "cellulase" shall be used herein to refer to any and all enzymes which catalyze the cleavage of cellulosic or lignocellulosic materials. As used herein, "cellulase" is synonymous with "cellulose-degrading enzymes." Explicitly, but not exclusively, included within the term cellulases are those enzymes which fall under the Enzyme Classification heading EC 3.2.1.x. A non-exhaustive list of these enzymes, the genes for all of which can be used in the present invention, includes the following:

Table 1: Polysaccharide-Degrading Enzymes

EC 3.2.1.1 (Alpha-amylase)
EC 3.2.1.2 (Beta-amylase)
EC 3.2.1.3 (Glucan 1,4-alpha-glucosidase)
EC 3.2.1.4 (Cellulase, also known as beta-1,4-endoglucanase, *e.g.*, cellulase E2)
EC 3.2.1.6 (Endo-1,3(4)-beta-glucanase)
EC 3.2.1.7 (Inulinase)
EC 3.2.1.8 (Endo-1,4-beta-xylanase)
EC 3.2.1.10 (Oligo-1,6-glucosidase)
EC 3.2.1.11 (Dextranase)
EC 3.2.1.14 (Chitinase)
EC 3.2.1.15 (Polygalacturonase)
EC 3.2.1.17 (Lysozyme)
EC 3.2.1.18 (Exo-alpha-sialidase)
EC 3.2.1.20 (Alpha-glucosidase)
EC 3.2.1.21 (Beta-glucosidase)
EC 3.2.1.22 (Alpha-galactosidase)
EC 3.2.1.23 (Beta-galactosidase)
EC 3.2.1.24 (Alpha-mannosidase)
EC 3.2.1.25 (Beta-mannosidase)
EC 3.2.1.26 (Beta-fructofuranosidase)
EC 3.2.1.28 (Alpha,alpha-trehalase)
EC 3.2.1.31 (Beta-glucuronidase)
EC 3.2.1.32 (Xylan endo-1,3-beta-xylosidase)
EC 3.2.1.33 (Amylo-1,6-glucosidase)
EC 3.2.1.35 (Hyaluronoglucosminidase)
EC 3.2.1.36 (Hyaluronoglucuronidase)
EC 3.2.1.37 (Xylan 1,4-beta-xylosidase)
EC 3.2.1.38 (Beta-D-fucosidase)
EC 3.2.1.39 (Glucan endo-1,3-beta-D-glucosidase)
EC 3.2.1.40 (Alpha-l-rhamnosidase)
EC 3.2.1.41 (Alpha-dextrin endo-1,6-alpha-glucosidase)
EC 3.2.1.42 (GDP-glucosidase)
EC 3.2.1.43 (Beta-L-rhamnosidase)
EC 3.2.1.44 (Fucoidanase)
EC 3.2.1.45 (Glucosylceramidase)
EC 3.2.1.46 (Galactosylceramidase)
EC 3.2.1.47 (Galactosylgalactosylglucosylceramidase)
EC 3.2.1.48 (Sucrose alpha-glucosidase)
EC 3.2.1.49 (Alpha-N-acetylgalactosaminidase)
EC 3.2.1.50 (Alpha-N-acetylglucosaminidase)
EC 3.2.1.51 (Alpha-L-fucosidase)
EC 3.2.1.52 (Beta-N-acetylhexosaminidase)

EC 3.2.1.53 (Beta-N-acetylgalactosaminidase)
EC 3.2.1.54 (Cyclomaltodextrinase)
EC 3.2.1.55 (Alpha-N-arabinofuranosidase)
EC 3.2.1.56 (Glucuronosyl-disulfoglucosamine glucuronidase)
EC 3.2.1.57 (Isopullulanase)
EC 3.2.1.58 (Glucan 1,3-beta-glucosidase)
EC 3.2.1.59 (Glucan endo-1,3-alpha-glucosidase)
EC 3.2.1.60 (Glucan 1,4-alpha-maltotetrahydrolase)
EC 3.2.1.61 (Mycodextranase)
EC 3.2.1.62 (Glycosylceramidase)
EC 3.2.1.63 (1,2-Alpha-L-fucosidase)
EC 3.2.1.64 (2,6-Beta-fructan 6-levanbiohydrolase)
EC 3.2.1.65 (Levanase)
EC 3.2.1.66 (Quercitrinase)
EC 3.2.1.67 (Galacturan 1,4-alpha-galacturonidase)
EC 3.2.1.68 (Isoamylase)
EC 3.2.1.70 (Glucan 1,6-alpha-glucosidase)
EC 3.2.1.71 (Glucan endo-1,2-beta-glucosidase)
EC 3.2.1.72 (Xylan 1,3-beta-xylosidase)
EC 3.2.1.73 (Licheninase)
EC 3.2.1.74 (Glucan 1,4-beta-glucosidase)
EC 3.2.1.75 (Glucan endo-1,6-beta-glucosidase)
EC 3.2.1.76 (L-iduronidase)
EC 3.2.1.77 (Mannan 1,2-(1,3)-alpha-mannosidase)
EC 3.2.1.78 (Mannan endo-1,4-beta-mannosidase)
EC 3.2.1.80 (Fructan beta-fructosidase)
EC 3.2.1.81 (Agarase)
EC 3.2.1.82 (Exo-poly-alpha-galacturonosidase)
EC 3.2.1.83 (Kappa-carrageenase)
EC 3.2.1.84 (Glucan 1,3-alpha-glucosidase)
EC 3.2.1.85 (6-Phospho-beta-galactosidase)
EC 3.2.1.86 (6-Phospho-beta-glucosidase)
EC 3.2.1.87 (Capsular-polysaccharide endo-1,3-alpha-galactosidase)
EC 3.2.1.88 (Beta-L-arabinosidase)
EC 3.2.1.89 (Arabinogalactan endo-1,4-beta-galactosidase)
EC 3.2.1.90 (Arabinogalactan endo-1,3-beta-galactosidase)
EC 3.2.1.91 (Cellulose 1,4-beta-cellobiosidase, also known as beta-1,4-exocellulases; cellobiohydrolases; and exoglucanases; *e.g.*, cellulase E3, CBH I)
EC 3.2.1.92 (Peptidoglycan beta-N-acetylmuramidase)
EC 3.2.1.93 (Alpha,alpha-phosphotrehalase)
EC 3.2.1.94 (Glucan 1,6-alpha-isomaltosidase)
EC 3.2.1.95 (Dextran 1,6-alpha-isomaltotriosidase)
EC 3.2.1.96 (Mannosyl-glycoprotein endo-beta-N-acetylglucosamidase)
EC 3.2.1.97 (Glycopeptide alpha-N-acetylgalactosaminidase)

EC 3.2.1.98 (Glucan 1,4-alpha-maltohexaosidase)
EC 3.2.1.99 (Arabinan endo-1,5-alpha-L-arabinosidase)
EC 3.2.1.100 (Mannan 1,4-beta-mannobiosidase)
EC 3.2.1.101 (Mannan endo-1,6-beta-mannosidase)
EC 3.2.1.102 (Blood-group-substance endo-1,4-beta-galactosidase )
EC 3.2.1.103 (Keratan-sulfate endo-1,4-beta-galactosidase)
EC 3.2.1.104 (Steryl-beta-glucosidase)
EC 3.2.1.105 (Strictosidin beta-glucosidase)
EC 3.2.1.106 (Mannosyl-oligosaccharide glucosidase)
EC 3.2.1.107 (Protein-glucosylgalactosylhydroxylysine glucosidase)
EC 3.2.1.108 (Lactase)
EC 3.2.1.109 (Endogalactosaminidase)
EC 3.2.1.111 (Mucinaminylserine mucinaminidase)
EC 3.2.1.111 (1,3-Alpha-L-fucosidase)
EC 3.2.1.112 (Deoxglucosidase)
EC 3.2.1.113 (Mannosyl-oligosaccharide 1,2-alpha-mannosidase)
EC 3.2.1.114 (Mannosyl-oligosaccharide 1,3-1,6-alpha-mannosidase)
EC 3.2.1.115 (Branched-dextran exo-1,2-alpha-glucosidase)
EC 3.2.1.116 (Glucan 1,4-alpha-maltotriohydrolase)
EC 3.2.1.117 (Amygdalin beta-glucosidase)
EC 3.2.1.118 (Prunasin beta-glucosidase)
EC 3.2.1.119 (Vicianin beta-glucosidase)
EC 3.2.1.120 (Oligoxyloglucan beta-glycosidase)
EC 3.2.1.121 (Polymannuronate hydrolase)
EC 3.2.1.122 (Maltose-6'-phosphate glucosidase)
EC 3.2.1.123 (Endoglycosylceramidase)
EC 3.2.1.124 (3-Deoxy-2-octulosonidase)
EC 3.2.1.125 (Raucaffricine beta-glucosidase)
EC 3.2.1.126 (Coniferin beta-glucosidase)
EC 3.2.1.122 (1,6-Alpha-L-fucosidase)
EC 3.2.1.128 (Glycyrrhizinate beta-glucuroniidase)
EC 3.2.1.129 (Endo-alpha-sialidase)
EC 3.2.1.130 (Glycoprotein endo-alpha-1,2-mannosidase)
EC 3.2.1.131 (Xylan alpha-1,2-glucuronosidase)
EC 3.2.1.132 (Chitosanase)
EC 3.2.1.133 (Glucan 1,4-alpha-maltohydrolase)
EC 3.2.1.134 (Difructose-anhydride synthase)
EC 3.2.1.135 (Neopullulanase)
EC 3.2.1.136 (Glucuronoarabinoxylan endo-1,4-beta-xylanase)
EC 3.2.1.137 (Mannan exo-1,2-1,6-alpha-mannosidase)
EC 3.2.1.138 (Anhydrosialidase)

DNA sequences encoding enzymes having any of the above-described functionalities can be obtained from several microbial sources, including bacterial and fungal sources. Cloning the gene or cDNA sequence of the desired enzyme can be achieved by several well-known methods. A preferred method is to purify the cellulase of interest (or purchase a sample if commercially available) and determine its N-terminal amino acid sequence, as well as several internal amino acid sequences, using known methods. Oligonucleotide probes corresponding to the amino acid sequence are then constructed (again using known methods) and used to screen a genomic or cDNA library of the organism from which the cellulase was isolated. Positive hybrids are identified, characterized using known methods (restriction enzyme analysis, etc.), and cloned by known means to yield DNA fragments containing the coding sequence for the desired cellulase activity. (See, for instance, *Current Protocols in Molecular Biology*, Chapters 5 and 6.)

If a partial nucleotide sequence of the cellulase of choice is already known, this information can be used to construct suitable primers to directly clone the corresponding cDNA using the polymerase chain reaction (PCR). (See *Current Protocols in Molecular Biology*, Chapter 15.)

Particularly preferred for use in the present invention are those enzymes falling within the classifications EC 3.2.1.4; EC 3.2.1.6; EC 3.2.1.21; and EC 3.2.1.91. The functionality of these particular enzymes is summarized as follows:

EC 3.2.1.4 enzymes ($\beta$-1,4-endoglucanases) hydrolyze internal 1,4 glycosidic bonds of the polysaccharide chain, thereby yielding new chain ends at the surface of cellulose crystals.

EC 3.2.1.6 enzymes ($\beta$-1,3-endoglucanases) hydrolyze internal 1,3 glycosidic bonds of the polysaccharide chain, which also results in the formation of new chain ends at the surface of cellulose crystals.

EC 3.2.1.21 enzymes ($\beta$-glucosidases) hydrolyze cellobiose into glucose, a readily fermentable substrate.

EC 3.2.1.91 enzymes ($\beta$-1,4-exocellulases) cleave cellobiosyl residues (cellobiose is a glucose dimer) from the chain ends of cellulose.

Particularly preferred enzymes (and hence particularly preferred genes) for use in the present invention are cellulase E2 and cellulase E3 of *T. fusca* and CBH I of *T. reesei*.

**Expression Constructs:**

Once the protein coding sequence (*i.e.*, the cellulase gene) has been identified and isolated, it must be inserted into an appropriate expression construct containing regulatory elements to direct the expression of the gene and to direct secretion of the gene product or targeting of the gene product to a particular sub-cellular location or organelle. Manipulation of oligonucleotide sequences using restriction endonucleases to cleave DNA molecules into fragments and DNA ligase enzymes to unite compatible fragments into a single DNA molecule with subsequent incorporation into a suitable plasmid, cosmid, or other transformation vector are well-known to the art.

A transcription regulatory sequence must be included in the expression construct in order to direct the transformed plant cells to transcribe the inserted cellulase coding sequence. Transcriptional regulators may be inducible or constituitive. Inducible transcription regulators direct transcription of the downstream coding sequences in a tissue-specific or growth-stage specific manner. Constituitive regulators provide for sustained transcription in all cell tissues. For purposes of the present invention, constructs which provide constituitive expression of the coding sequence are preferred.

It is also preferred that the expression construct contain a transcription initiation sequence from the tumor-inducing plasmid (Ti) of *Agrobacterium*. Several T-DNA transcription initiation sequences are well known and include, without limitation, the octopine synthase, nopaline synthase, and mannopine synthase initiators.

Downstream of the initiation sequence and fused to the coding sequence, the expression construct may be manipulated to contain a leader signal sequence which directs the resulting polypeptide to a particular organelle or targets the expressed product for secretion (or to signal post-transcriptional or post-translational modification of the gene product).

Likewise, the expression construct should also include a termination sequence to signal transcription termination.

To facilitate selection of successfully transformed plants, the expression construct should also include one or more selectable markers. The neomycin phosphotransferase gene (NPT II), a well-characterized and widely employed antibiotic resistance selection marker is preferred. This marker provides resistance to kanamycin. A large number of other markers are known and can be used with equal success (*e.g.*, other antibiotic resistance markers, dihydrofolate reductase, luciferase, β-glucuronidase, and the like).

Figs. 1 and 2 depict schematic representations of suitable expression constructs for transformation of plants. These constructs are intended for use with *Agrobacterium*-mediated transformation using the binary vector approach. However, these same constructs can be coated onto micro-projectiles for transformation by particle bombardment. With the exception of the coding sequence, these two constructs are essentially identical: Fig. 1 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E2 of *T. fusca*.
Fig. 2 is a schematic diagram of binary vector T-DNA for an expression construct to transform plants to contain cellulase E3 of *T. fusca*.

In both Fig. 1 and Fig. 2, promoters and structural genes are depicted as arrows which indicate the direction of transcription and terminators are depicted as boxes. See the "Brief Description of the Figures" for a legend to the abbreviations. In the expression constructs depicted in Figs. 1 and 2, the "MAC" hybrid promoter drives the transcription of the recombinant cellulase genes. Both constructs also contain a constituitive NPT II expression cassette to allow for antibiotic resistance selection using kanamycin. The coding sequence of the construct shown in Fig. 1 (Met-E2m) encodes cellulase E2 from *T. fusca*. (See SEQ. ID. NO: 1; ATG start codon at nt's 255-257, TGA stop codon at nt's 1578-80, first codon of mature E2 protein (AAT) at nt's 348-350.) This sequence encodes the mature form of the enzyme with an N-terminal methionine added. In the same fashion, the coding sequence of the construct shown in Fig. 2 (Met-E3m) encodes cellulase E3 from *T. fusca*. (See SEQ. ID. NO: 2; ATG start

codon at nt's 575-577, TAA stop codon at nt's 2363-65, first codon of mature E3 protein (GCC) at nt's 689-692.) This sequence also encodes the mature form of the enzyme with an N-terminal methionine added. (See the Examples, below.)

**Transformation of Plants:**

Transformation of the plants can be accomplished by any means known to the art, including *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and virus-mediated transformation. The method of transformation is not critical to the functionality of the present invention insofar as the method chosen successfully incorporates the oligonucleotide construct containing the cellulase-encoding region and any accompanying regulatory sequences into the plant host. The nature of the plant host to be transformed has some bearing on the preferred transformation protocol. For dicots, *Agrobacterium*-mediated transformation utilizing protoplasts or leaf disks is most preferred. Although the Examples disclose the use of tobacco and alfalfa as bioreactors for cellulase production, any crop plant, including monocots, can be utilized. Transformation of monocots is typically achieved by particle bombardment of embryogenic cell lines or cultured embryos. See, for instance, *Vasil et al.* (1993) and *Castillo et al.* (1994). Recent developments in "super-binary" vectors, however, also allow for the use of *Agrobacterium*-mediated gene transfer in most of the major cereal crops. See, for instance, *Ishida et al.* (1996). In this case, the explant source is typically immature embryos.

*Agrobacterium*-mediated transformation of the plant host using explants is preferred for its relative ease, efficiency, and speed as compared to other methods of plant transformation. For example, disks are punched from the leaves of the plant host and cultured in a suitable medium where they are then exposed to *Agrobacterium* containing the expression construct and (preferably) a disarmed tumor-inducing (Ti) plasmid. *Agrobacterium tumefaciens* LBA 4404 is the preferred strain for transformation. The preferred binary vector is the pCGN1578 binary vector (*McBride and Summerfelt* (1990)).

The binary vector transformation method is well known and needs only be briefly described herein. See *Zambryski et al.* (1989) for a complete review. The Ti plasmid of *Agrobacterium* contains virulence genes (*vir*) which encode trans-acting proteins that enable the transfer of a portion of the plasmid (the T-DNA) to a plant cell. The T-DNA portion of the Ti plasmid is flanked by two border regions (the right and left borders) which act as recognition sites for the excision of the T-DNA from the plasmid prior to its transfer to the plant host. Excision of the T-DNA is mediated by the *vir* genes of the Ti plasmid and involves nicking of the right and left borders of the T-DNA, which frees a single-stranded oligonucleotide fragment. This fragment is then mobilized out of the *Agrobacterium* and into the plant host target.

In the binary vector method, the T-DNA with its right and left border regions is cloned into *E. coli* in known fashion, and the wild-type genes normally found between the two border regions is excised. The expression construct encoding the cellulase of interest is inserted between the right and left border regions. This construct is designated the "binary plasmid." Construction of the binary plasmid is accomplished utilizing the well-characterized recombinant genetic methods applicable to *E. coli*. Successful transformants are selected utilizing a co-transformed marker appropriate for *E. coli*.

The binary plasmid is then mobilized back into *Agrobacterium*. This is accomplished by direct transformation procedures well known to those skilled in the art.

The *Agrobacterium* itself, such as the preferred LBA 4404 strain, is genetically manipulated to contain a Ti plasmid (called the helper plasmid) which lacks the T-DNA and the tumor-inducing regions (i.e., the Ti plasmid is "disarmed") but which still encodes the virulence proteins necessary for DNA transfer. By cooperation between the helper plasmid and the binary plasmid, the length of DNA between the two border regions of the binary plasmid is excised and mobilized into the plant host, where it is incorporated into the plant host genome. The binary method derives its name from the fact that the plasmid containing the expression construct to be transferred is maintained within *Agrobacterium* as a distinct and independently replicating vector from the Ti plasmid itself.

Selection of successful transformants is accomplished using the co-transformed selection marker discussed above. If the marker is NPT II, selection is accomplished by treatment with kanamycin.

For the present invention, the most preferred plants for transformation are alfalfa and tobacco. However, any plant species will function with comparable success. Included among the plant species which can be utilized in the present invention are cauliflowers, artichokes, apples, bananas, cherries, cucumbers, grapes, lemons, melons, nuts, oranges, peaches, pears, plums, strawberries, tomatoes, cabbages, endive, leeks, lettuce, spinach, arrowroot, beets, carrots, cassava, turnips, radishes, yams, sweet potatoes, beans, peas, soya, wheat, barley, corn, rice, rapeseed, millet, sunflower, oats, tubers, kohlrabi, potatoes, and the like.

The plants to be transformed are preferably common green field plants, such as the preferred alfalfa and tobacco, as well as soya, corn, and the like. Equally preferred are plant hosts which are grown specifically for "biomass energy," such as switchgrass, poplar, and the like. In this instance, the enzymes would not be recovered from the plants. The plants are then transformed and regenerated into whole plants which express fully-functional, cellulose-degrading enzymes in economically significant quantities.

Alfalfa is one of the most preferred plant species for use in the present invention because alfalfa is a hardy, perennial plant, which grows well with minimal fertilization and irrigation. Alfalfa is also a very prolific plant. In temperate areas such as those found in the midwestern United States, alfalfa will yield three or more harvests per growing season. Methods have also been developed for wet fractionation of the herbage matter to recover value-added products therefrom.

Tobacco is equally preferred for its prolific growth, ease of transformation, and its well-characterized genetics. Both alfalfa and tobacco are widely cultivated throughout the United States and in other parts of the world.

In the most preferred embodiment, alfalfa or tobacco plants are stably transformed to express, constituitively, enzymatically active E2 or E3 cellulases from *T. fusca*. Also preferred are alfalfa or tobacco which express enzymatically active CBH I from *T. reesei* or combinations of E2, E3, and CBH I. The *T. fusca* cellulases are most preferred

because they are native to thermo-tolerant bacteria and are relatively heat stable. This allows isolation of the cellulase from plant material using relatively rigorous heat precipitation without adversely effecting the activity of the cellulase.

**Stage-Specific and Tissue-Specific Expression of Cellulases:**

Because the enzymes to be expressed by the transformed plant hosts hydrolyze components of the plant cell wall, high levels of expression might have a deleterious effect on the plant host. Therefore, targeting of the expressed enzyme to particular sub-cellular compartments may be preferred. Targeting of the expressed enzyme may also be preferred to avoid expression of the enzyme in sub-cellular compartments where proteolytic activity is high. Targeting of the expressed enzyme may also be preferred if the exogenous cellulase activity interferes with the normal cellular metabolism of certain compartments.

For instance, targeting expression to the apoplast allows the exogenous protein to avoid the more active protein-degrading systems of other cellular compartments, such as in plant leaf vacuoles.

Several signal sequences are known and can be utilized in the present invention. For example, signal sequences for targeting to the secretory pathway are known, *Wandelt et al.* (1992), *Bednarek* (1991), *Mason et al.* (1988), as are sequences for targeting to the chloroplast, *Keegstra et al.* (1993), and the mitochondrion, *de Castro Silva Filho et al.* (1996).

For apoplast targeting, the VSP leader is preferred. The VSP leader comprises the amino acid sequence: $NH_3$-Met-Lys-Leu-Phe-Val-Phe-Phe-Val-Ala-Ala-Val-Val-Leu-Val-Ala-Trp-Pro-Cys-His-Gly-Ala- (SEQ. ID. NO: 3). See *Mason et al.* (1988).

Additionally, bacterial secretory sequences found in the wild-type cellulase gene may be removed to afford cytoplasmic expression of the enzyme in the recombinant plant host.

Targeting can be achieved by fusing combinations of mitochondrial and chloroplast targeting signals to the N-terminus of the desired cellulase, as has been

demonstrated for the reporter genes chloramphenicol acetyl transferase and β-glucuronidase, *de Castro Silva Filho et al.* (1996). In some cases, efficient translocation requires the presence of both signal peptides, with the amino terminal peptide being crucial in specifying import into a particular organelle. In addition, vacuole targeting can be achieved by fusing the sequence encoding the N-terminal 146 amino acids of the vacuolar patatin protein between a secretory leader and structural gene for the cellulase, as has been demonstrated for the yeast invertase gene, *Sonnewald et. al.* (1991).

**Regeneration of Mature Transgenic Plants:**

Transgenic tobacco and alfalfa were produced by *Agrobacterium*-mediated transformation using explants as source material. This is a routine method easily followed by those skilled in the art. The production methods for transgenic tobacco and alfalfa are given as non-limiting illustrations of the practice of the invention.

The transformation procedure for tobacco is essentially the explant method developed by Horsh et al. (1985). Leaf explants are taken from the second and third fully expanded leaves of three-week old *in vitro* shoot cultures of *Nicotiana tabacum* W38 maintained on MS medium, *Murashige and Skoog* (1962). The leaf pieces are cut into 1 cm squares and pre-cultured on MS medium with 2.0 mg/L 6-benzyl-aminopurine (BAP) and 0.1 mg/L alpha-naphthalene acetic acid (NAA) for 24 hours at 25°C with a 16 hour photo period of 70-90 $\mu E\ m^{-2}s^{-1}$. After pre-culture, explants are placed into a suspension of *Agrobacterium* cells. After 30 minutes, leaf explants are blotted on filter paper and placed abaxial-side down on MS medium with 1.0 mg/L BAP and 0.1 mg/L NAA and co-cultivated for four days under the same conditions as given above. Leaf pieces are then rinsed three times in sterile water, blotted on filter paper, and transferred to the media used for co-cultivation but containing 100 mg/L kanamycin and 400 mg/L carbenicillin. Plantlets (typically 2-3) develop 10-14 days later from callus formed along cut leaf edges. If desired, further plantlet formation can be achieved by transfer of explants to fresh medium at two week intervals. Plantlets are excised and rooted on MS media containing 100 mg/L kanamycin and 400 mg/L carbenicillin.

To transform alfalfa, new-growth trifoliates are taken from alfalfa plants (regenerable genotypes, *Bingham et al.* (1975)) maintained in a growth room and sterilized using alcohol and bleach washes (30 seconds in 70% alcohol, 90 seconds in 20% hypochlorite + 0.1% SDS, followed by three rinses in sterile distilled water). Leaf edges are cut on moist filter paper and tissue then placed into liquid SH-II medium. (*Bingham et al., supra.*) When sufficient explants have been taken, the explants are moved to a suspension of *Agrobacterium* cells containing the engineered plasmid. (The *Agrobacterium* suspension is taken from an overnight culture grown in liquid YEP selection medium.) Cell density is adjusted to fall between about 0.6 to about 0.8 at $A_{660}$. After 30 minutes inoculation, the explants are gently blotted on filter paper and placed on B5H medium, *Brown and Atanassov* (1985), for four days. They are then rinsed twice in sterile water and cultured on B5H for a further four days. At the end of this period, they are rinsed three times and transferred to B5H containing 25 mg/L kanamycin and 250 mg/L carbenicillin. Plates are maintained at 24°C, 16 hour photo period, light intensity 60-80 $\mu E\ m^{-2}s^{-1}$. Explant-derived calli (and occasionally embryoids) which form within 3 weeks on this medium are moved to B5H with antibiotics but without hormones to allow for further embryoid production and development of existing embryoids. After three to four weeks, embryos are transferred to MS medium including the two antibiotics to allow for development into plantlets. Callus forms on untreated explants in the presence of 25 mg/L kanamycin but embryos are never produced. Each explant piece can give rise to multiple (up to 40) embryos. Plantlets are rooted on MS medium lacking antibiotics.

**Monitoring Cellulase Expression:**

Cellulase expression can be monitored using a number of different methods, the two most common being western blot analysis (which detects cellulase protein using antibodies specific for the cellulase of interest) and zymographic analysis or enzyme assay (both of which measure the ability of the expressed cellulase to degrade a cellulosic substrate).

Briefly, in the western blot technique, whole plant samples (or root tips, leaves, etc.) are ground in an extraction buffer (preferably 50 mM sodium acetate (pH 5.5) and 10 mM dithiothreitol) and an aliquot of the extract loaded onto an electrophoresis gel (*e.g.*, polyacrylamide containing SDS). Preferably, identical extractions are performed on non-transformed plants and aliquots of these extractions are then loaded onto parallel lanes of the gel to act as negative controls. Serial dilutions of purified cellulase standards can be also electrophoresed to act as positive controls. The gel is then subjected to electrophoresis in standard and well known fashion.

After electrophoresis is complete, the separated proteins are electro-transferred to a nitrocellulose, PVDF, or nylon membrane, in well known fashion. The membrane containing the immobilized proteins is then immersed in a non-specific blocking buffer or detergent (*e.g.*, "TWEEN 20"), and then placed in a solution containing an antibody (the primary antibody) which is specifically reactive with the particular cellulase under investigation. The membrane is then washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (*e.g.*, goat anti-rabbit IgG). The membrane is then exposed to a chromogenic or luminescent substrate to visualize cellulase hybridization on the membrane.

Zymograms in which the cellulase of interest is resolved in a gel system and then assayed for activity within the gel provide a relatively simple way to assess the activity of cellulases in crude cell lysates. See *Coughlan* (1988). In this approach, plant tissue is ground in the presence of an appropriate grinding buffer (100 mM Tris-HCl pH 9.0, 5 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM ethylenediamine-tetraacetic acid, for example). After grinding of the tissue, an equal volume of a 50% (v/v) slurry of washed polyvinylpolypyrrolidone (suspended in grinding buffer) is added and mixed thoroughly. After centrifugation of the mixture, a sample of the cleared extract is subjected to electrophoresis through a non-denaturing (8%, w/v) polyacrylamide gel. The resulting gel is used to prepare a sandwich with a thin film (<2 mm) of agarose (0.7 % agarose, 0.5 % Sigma medium viscosity carboxymethycellulose) bonded to "GELBOND" film (FMC Corporation). After incubation for 1.5 hours at 50° C, the agarose film is stained with "CONGO RED" dye

for 30 minutes followed by a 1M NaCl wash. After several minutes, it is possible to visualize cellulase activity as a clear zone within a background of red staining.

Cellulase activity is most commonly assayed in aqueous solution, using a cellulosic substrate and monitoring the reaction for either the release of a chromophore/fluorophore or release of cellobiose ("reducing sugar"). For example, *T. fusca* E2 activity can be measured by incubating a sample of the enzyme in a 0.4 ml reaction containing 1% (w/v) low viscosity carboxymethylcellulose (Sigma C-5678) and 50 mM NaOAc pH 5.5 at 55°C for 2-20 hours. 1.0 ml of DNS solution, see *Irwin et al.* (1993), is then added and the mixture is boiled for 15 minutes. Measurement of absorbance values at 600 nm for each reaction can then be correlated to values determined for a known series of glucose standards to determine the extent of carboxymethylcellulose hydrolysis. For plant extracts, background values are determined by preparing parallel reaction samples which contain no substrate and subtracting this value from that obtained in the presence of 1% carboxymethylcellulose.

For a more complete discussion of cellulase assays, see *Adney et al.* (1994), *Baker et al.* (1992), *Tucker et al.* (1989) and *Irwin et al.* (1993).

**Isolation of Cellulase Activity from Plants:**

It is most preferred that, where applicable, the enzyme not be purified from the plant material, but rather that the plant material containing the cellulase activity be used directly. This is demonstrated in the Examples, below, where transgenic alfalfa which expressed cellulase activity is added directly to silage materials to further the extent of fermentation.

If isolation of the cellulase activity is desired, this can be accomplished by any means known to the art. For example, the preferred *T. fusca* E2, E3, and CBH I enzymes are taken from thermo-tolerant bacteria. The activity of these enzymes remains unchanged by treatments up to about 55-60°C. Therefore, these enzymes can be isolated by gently heating the plant material in aqueous buffered solution (100 mM Tris/HCl pH 9.0, for example) to precipitate the bulk of plant proteins. The soluble cellulase enzymes

are then recovered and further purified by any means known to the art, including HPLC, affinity chromatography, and the like. To facilitate downstream processing of the enzyme, a purification tag may optionally be incorporated into the expressed cellulase.

Since the above-mentioned enzymes are well-characterized, the preferred purification scheme is based on established protocols already in existence. For example, *T. fusca* E2 from a heat-treated plant extract is further purified by adsorption to a phenyl "SEPHAROSE" column in the presence if 0.8 M ammonium sulfate. Successive column washes using ammonium sulfate concentrations of 0.6 M and 0.3 M in a buffer containing 5 mM KPi, pH 6.0 and 5 mM NaCl are followed by a final wash with 0.1 M KPi, pH 6.0. Elution of E2 is accomplished using 5 mM KPi, pH 6.0. Peak fractions are loaded on a hydroxylapatite column (equilibrated with 1 mM KPi, pH 6.0) and the flow-through fractions collected and pooled. The pooled fractions are loaded on a "Q-SEPHAROSE" column (pre-equilibrated with 10 mM BisTris, pH 6.0) and eluted with a continuous buffer gradient from 5 mM BisTris, pH 6.0 to 150 mM NaCl, 5 mM BisTris, pH 6.0. Peak fractions from the "Q-SEPHAROSE" column are then pooled, concentrated and stored frozen in convenient aliquots. Similarly detailed protocols exist for both *T. fusca* E3 and *T. reesei* CBH I, see *Irwin et al.* (1993).

## EXAMPLES

The following Examples are included solely to aid in a more complete understanding of the manufacture and use of the transgenic plants disclosed and claimed herein. The Examples do not limit the scope of the invention in any fashion.

### Example 1: Production of Transgenic Alfalfa and Tobacco Which Express Cellulase "E2" of *T. fusca*

Transgenic alfalfa and tobacco plants were produced using the same protocol. Binary vectors carrying recombinant cellulase expression cassettes were transformed into *Agrobacterium tumefaciens* strain LBA 4404, facilitating *Agrobacterium*-mediated

transformation of plant tissue. The construct used is shown in Figure 1. The gene encoding the E2 cellulase of *Thermomonospora fusca* was obtained as described by *Ghangas & Wilson* (1988). The E2 gene was modified by PCR using the XbaE2 primer, 5'-GC*TCTAGA*ATGAATGATTCTCCGTTC-3' (SEQ. ID. NO: 4) and the "-20 sequencing primer," 5'-TGACCGGCAGCAAAATG-3' (SEQ. ID. NO: 5), (product #1211, New England Biolabs, Inc., Beverly, Massachusetts), resulting in a recombinant gene in which an *Xba I* site (***bold italics***) was incorporated immediately 5' to an introduced start codon (underlined). This start codon precedes the first codon encoding the mature form of the E2 protein (AAT, nt's 348-350 in SEQ. ID. NO: 1). The net effect of these changes is the removal of the bacterial secretion signal peptide (resulting in cytosolic accumulation), the addition of a novel cloning site to facilitate expression cassette construction and the addition of a methionine residue to the N-terminus of the protein compared to the processed mature form of E2 obtained from *T. fusca*).

The cloned E2 gene required no modification at the 3' end as a convenient *Eco RI* restriction site occurs approximately 45 nucleotides 3' to the stop codon.

The preferred expression cassette includes the hybrid "MAC" promoter and the mannopine synthetase terminator. The MAC promoter contains distal elements, including the transcriptional enhancer, of the CaMV 35S promoter (-940 to -90, relative to the mRNA start site), as well as proximal promoter elements derived from the *Agrobacterium* mannopine synthetase promoter (-301 to +65 relative to the mRNA start site). MAC has been reported to result in higher levels of expression than either of the natural promoters (*Comai et al.* (1990).) The expression cassette was cloned into the pCGN1578 binary vector and mobilized into *Agrobacterium*.

Initial tobacco transformants were screened by western blot to determine the level of expression. Levels of expression ranged up to 0.1 to 0.2 % of extracted protein. The mature plants were allowed to self and set seed. One of the initial transformants, designated CT30, was tested further to verify the sexual transmission of the transgene. S1 seeds from this plant were germinated and tested for kanamycin resistance. Leaf samples from $kan^R$ seedlings as well as a W38 control were prepared for western blot

analysis as described previously. The results are depicted in Fig. 3. Each lane contained extract corresponding to 5 mg fresh weight of leaf tissue. In addition, 1 ng of purified E2 enzyme was loaded as a control. Levels of expression were similar to that observed in the parental transformant, demonstrating the stable sexual transfer of this trait. Similar genetic stability was also observed in alfalfa plants transformed with this transgene.

The thermal stability and enzymatic activity of recombinant *T. fusca* E2 was demonstrated using transgenic alfalfa. Samples were prepared for zymogram analysis as described above. As shown in Fig. 5, aliquots of alfalfa extract were treated for 5, 10 and 20 minutes (grouped from left to right) at each of the temperatures indicated (°C) before being subjected to native gel electrophoresis. An untreated sample of extract and two purified E2 standards were included as controls. Levels of E2 activity corresponded well with expected activity based on western blot analysis of samples from the same plant. In addition, no significant loss in band intensity (activity) was observed at any of the treatment temperatures, despite the fact that greater than 95% of the soluble protein in the extract is denatured after 20 minutes at 65°C.

**Example 2: Production of Transgenic Alfalfa and Tobacco Which Express Cellulase "E3" of *T. fusca***

Here, the expression construct depicted in Fig. 2 was used to transform alfalfa and tobacco using the same methodology as described in Example 1.

The gene encoding the E3 cellulase of *Thermomonospore fusca* was obtained as described by *Zhang et al.* (1995). The 5' end of the E3 gene was modified by PCR using the primer XbaE3, 5'-GC***TCTAGA***ATGGCCGGCTGCTCGGTG-3' (SEQ. ID. NO: 6), resulting in a recombinant gene in which an ***Xba I*** site (***bold italics***) was incorporated immediately 5' to an introduced start codon (underlined). This start codon precedes the first codon encoding the mature form of the E3 protein (GCC, nt 689-691 in SEQ. ID. NO: 2). The 3' end of the E3 gene was modified using the primer RIE3, 5'-G*GAATTC*TTACAGAGGCGGGTAG-3' (SEQ. ID. NO: 7), thereby placing an *Eco RI*

restriction site (***bold italics***) immediately 3' to the stop codon (<u>underlined</u>) for the E3 gene. Note that this latter primer is homologous to the noncoding strand of the E3 gene. The net effect of these changes is the removal of the bacterial secretion signal peptide (resulting in cytosolic accumulation), the addition of novel cloning sites to facilitate expression cassette construction and the addition of a methionine residue to the N-terminus of the protein (compared to the processed, mature form of E3 obtained from *T. fusca*).

The E3 expression cassette was constructed as described above for the E2 cassette.

Initial tobacco transformants were screened by western blot to determine the level of expression. Levels of expression ranged up to about 0.04% of extracted protein. The mature plants were allowed to self and set seed. One of the initial transformants, designated CT117, was tested further to verify the sexual transmission of the transgene. S1 seeds from this plant were germinated and tested for kanamycin resistance. Leaf samples from $kan^R$ seedlings as well as a W38 control were prepared for western blot analysis as described previously. The results are depicted in Fig. 4. Each lane contained extract corresponding to 5 mg fresh weight of leaf tissue. In addition, 1 ng of purified E3 enzyme was loaded as a control. Levels of expression were comparable to that observed in the parental transformant, demonstrating the stable sexual transfer of this trait.

**Example 3: Sexual Transfer of Cellulase Expression in Tobacco and Alfalfa**

Original transgenic lines of tobacco and alfalfa shown to express either E2 or E3 cellulase were used in sexual crosses. In both cases, the trait segregated in progeny as predicted by Mendelian genetics. Expression levels were the same as, or greater than those seen in parental lines.

Example 4: Use f Transgenic Alfalfa in Silage

Here, regular non-transformed alfalfa, alfalfa transformed according to Example 1 and alfalfa transformed according to Example 2 were ensiled under identical conditions for one month and the products of fermentation for each experiment quantified. The results are presented in Table 2.

All of the ensiled plant material was ground separately through a manual meat grinder. The grinder was rinsed with water and wiped with ethanol after grinding each sample. A 1 to 1 to 1 mixture of non-transformed alfalfa, E2-transformed alfalfa, and E3-transformed alfalfa was ground together and used to assemble two control silos (Cont1 and Cont2, 50 g each). The two control silos were inoculated with a 1 mL of a commercial inoculant (0.1098 g "BIOMATE LP/PC" concentrate in 500 mL sterile water).

Two silos each of E2-transformed alfalfa (E2-1, E2-2) and E3-transformed alfalfa (E3-1, E3-2) were constructed in the same fashion as the controls (35 g each, inoculated with 0.6 mL of the above-noted inoculant). Two silos of mixed E2- and E3-transformed alfalfa were constructed by grinding together 17.5 g each of E2- and E3-transformed alfalfa per silo (35 g each, inoculated with 0.6 mL of the above-noted inoculant).

All of the silos were then placed into a 30°C water bath until opening.

Of special note in this Example is the increased amount of fermentation products in the transgenic alfalfa as compared to the non-transformed alfalfa. In particular, note that a mixture of alfalfa herbage expressing both the E2 and E3 cellulases exhibits markedly improved fermentation yield as compared to the non-transformed alfalfa and ensiled alfalfa expressing either E2 or E3 enzymes.

Clearly, as shown by this Example, expression of cellulases in transgenic alfalfa leads to better silage production.

The invention is not limited to the preferred embodiments, transformation protocols, transformed plant hosts, and expression constructs explicitly described above, but encompasses all such forms thereof as are encompassed within the scope of the attached claims.

TABLE 2

| | | | | | Organic Acid Analysis (OAA) via HPLC | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ID | %DM | pH | SUC | LAC | FOR | ACE | PRO | 2,3But | ETOH | BUT | Total Prod. | Avg. Total Prod. |
| Cont 1 | 23.33612 | 5.741 | 0.144 | 2.014 | 0.000 | 1.764 | 0.000 | 0.239 | 0.704 | 0.000 | 4.86 | 5.47 |
| Cont 2 | 21.62983 | 5.121 | 0.159 | 3.885 | 0.000 | 1.407 | 0.328 | 0.000 | 0.294 | 0.000 | 6.07 | |
| E2-1 | 22.99369 | 5.277 | 0.157 | 3.390 | 0.000 | 2.300 | 0.233 | 0.000 | 0.330 | 0.000 | 6.41 | 6.09 |
| E2-2 | 23.83774 | 5.166 | 0.361 | 2.935 | 0.000 | 1.998 | 0.177 | 0.000 | 0.298 | 0.000 | 5.77 | |
| E3-1 | 22.88773 | 5.128 | 0.283 | 3.321 | 0.000 | 2.380 | 0.177 | 0.000 | 0.292 | 0.000 | 6.45 | 6.61 |
| E3-2 | 22.22822 | 5.151 | 0.354 | 3.324 | 0.000 | 2.608 | 0.200 | 0.000 | 0.288 | 0.000 | 6.77 | |
| E23-1 | 22.95945 | 5.743 | 0.551 | 2.848 | 0.000 | 3.185 | 0.328 | 0.000 | 0.337 | 0.000 | 7.25 | 7.45 |
| E23-2 | 22.66411 | 5.888 | 0.602 | 2.745 | 0.000 | 3.649 | 0.301 | 0.000 | 0.353 | 0.000 | 7.65 | |

The table headings are as follows:

%DM = percent dry matter of silage, pH = acidity, SUC = succinic acid, LAC = lactic acid, FOR = formic acid, ACE = acetic acid, PRO = propionic acid, 2,3 But = 2,3-butanediol, ETOH = ethanol, BUT = butyric acid, Cont 1 and Cont 2 = controls, E2-1 and E2-2 = transformant expressing E2, E3-1 and E3-2 = transformants expressing E3, E23-1 and E23-2 = a 1:1 mixture of herbage from transgenic alfalfa expressing E2 and E3.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Austin-Phillips, Sandra
Burgess, Richard R.
German, Thomas L.
Ziegelhoffer, Thomas L.

(ii) TITLE OF INVENTION: Transgenic Plants as an Alternative Source of Lignocellulosic-Degrading Enzymes

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DeWitt Ross & Stevens, S.C.
(B) STREET: 8000 Excelsior Drive, Ste. 401
(C) CITY: Madison
(D) STATE: Wisconsin
(E) COUNTRY: U.S.A.
(F) ZIP: 53717-1914

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/028,718
(B) FILING DATE: 17-OCT-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sara, Charles S.
(B) REGISTRATION NUMBER: 30,492
(C) REFERENCE/DOCKET NUMBER: 09820.036

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (608) 831-2100
(B) TELEFAX: (608) 831-2106

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1621 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Thermomonospora fusca cellulase E2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGATATGGAT GATCTGACGT CTGAATCCCC TTGTCACCCT AGACATTCAC CCATTTTGTC 60

GCTTTTACGG CTTTCTTTGG GAGTTCTCCG TTTCACCAAG GAACAAAACC GCAACGGAGA 120

GTAGGCGCGG TCTTTACAGC TCCCTTGCCA ATGGTTATCG TCCGAACGGA AAACGATCTG 180

GGAGCGCTCC CAGCCATGCG CTCCTCTTCG TGCCCCTCAC TTCTTTTGAG CCTTGTGCTC 240

GTTAGGAGCC CCGAATGTCC CCCAGACCTC TTCGCGCTCT TCTGGGCGCC GCGGCGGCGG 300

CCTTGGTCAG CGCGGCTGCT CTGGCCTTCC GGTCGCAAGC GGCGGCCAAT GATTCTCCGT 360

TCTACGTCAA CCCCAACATG TCCTCCGCCG AATGGGTGCG GAACAACCCC AACGACCCGC 420

GTACCCCGGT AATCCGCGAC CGGATCGCCA GCGTGCCGCA GGGCACCTGG TTCGCCCACC 480

ACAACCCCGG GCAGATCACC GGCCAGATCG ACGCGCTCAT GAGCGCCGCC CAGGCCGCCG 540

GCAAGATCCC GATCCTGGTC GTGTACAACG CCCCGGGCCG CGACTGCGGC AACCACAGCA 600

GCGGCGGCGC CCCCAGTCAC AGCGCCTACC GGTCCTGGAT CGACGAATTC GCTGCCGGAC 660

TGAAGAACCG TCCCGCCCAC ATCATCGTCG GGCCGGACCT GATCTCGCTG ATGTCGAGCT 720

GCATCCAGCA CGTCCAGCAG GAAGTCCTGG AGACGATGGC GTACGCGGGC AAGGCCCTCA 780

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CCGTGACATG GACCTTCACC GACGGCCAGA CCATCACCAA CGCCTGGAAC GCCGACGTGT 1440

CCACCAGCGG CTCCTCGGTG ACCGCGCGGA ACGTCGGCCA CAACGGAACG CTCTCCCAGG 1500

GAGCCCCCAC AGAGTTCGGC TTCGTCGGCT CTAAGGGCAA CTCCAACTCT GTTCCGACCC 1560

TTACCTGCGC CGCCAGCTGA CCCCTCCTGG CAGTGCACTG GGTGGCTTAG GCGTGCTGGG 1620

G 1621

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3503 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Thermomonospora fusca cellulase E3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGCGATCCC CCTCATCATT CAGGTGCGGT TAGTTCCCCC AGGCTACCGA GGACCGAATT 60

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CTTGCGCGTC CCGGCGGAGG GGGGAAGCAC CCCGCGAGAT GGCTCCGCCA CGGCCTGTTT 180

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CTCTCTTTTG GGAGCGCTCC CGTGCCGCTA GTCACACTGG GACGTGAATG GCGTCACGGT 300

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GCAACCACCC CGACCGGTCG GCTGAACACT GCAGCGTCCG GTTCTCGACC ATCCCCTTGC 540

GAGAGAACAT CCTCCAACCA AGGAAGACAC CGATATGAGT AAAGTTCGTG CCACGAACAG 600

ACGTTCGTGG ATGCGGCGCG GGCTGGCAGC CGCCTCTGGA CTGGCGCTTG GCGCCTCCAT 660

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: VSP Leader Sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

| Met | Lys | Leu | Phe | Val | Phe | Phe | Val | Ala | Ala | Val | Val | Leu | Val | Ala | Trp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | 5 | | | | | 10 | | | | | 15 | |

| Pro | Cys | His | Gly | Ala |
|---|---|---|---|---|
| | | | 20 | |

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Xba E2 PCR Primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGACCGGCAG CAAAATG 17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTCTAGATG GCCGGCTGCT CGGTG 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAATTCTTA CAGAGGCGGG TAG 23

## CLAIMS

What is claimed is:

1. A genetically recombinant plant comprising a plant transformed to contain one or more exogenous gene sequences or fragments thereof which encode one or more cellulose-degrading gene products and wherein the gene product or products are classified within an enzyme classification selected from the group consisting of EC 3.2.1.4, EC 3.2.1.6, EC 3.2.1.21, EC 3.2.1.91, and combinations thereof, and wherein the gene product or products are expressed in recoverable quantities.

2. The genetically recombinant plant of Claim 1, wherein the gene product or products are expressed constituitively.

3. The genetically recombinant plant of Claim 1, wherein the gene product or products are expressed stage-specifically.

4. The genetically recombinant plant of Claim 1, wherein the gene product or products are expressed tissue-specifically.

5. The genetically recombinant plant of Claim 4, wherein the gene product or products are expressed in a plant tissue selected from the group consisting of seeds, fruit, stems, leaves, and tubers.

6. The genetically recombinant plant of Claim 4, wherein the plant contains at least two exogenous genes and wherein their respective gene products are expressed independently of one another.

7. The genetically recombinant plant of Claim 1, wherein the gene product or products are expressed in a targeted sub-cellular compartment.

8. The genetically recombinant plant of Claim 7, wherein the gene product or products are expressed in a sub-cellular compartment selected from the group consisting of: plastid, cytosol, endoplasmic reticulum, mitochondrion, inclusion body, and vacuole.

9. The genetically recombinant plant of Claim 7, wherein the plant contains at least two exogenous genes and wherein their respective gene products are expressed independently of one another.

10. The genetically recombinant plant of Claim 1, wherein the gene product or products are expressed extra-cellularly.

11. The genetically recombinant plant of Claim 1, wherein the plant is dicotyledonous.

12. The genetically recombinant plant of Claim 1, wherein the plant is monocotyledonous.

13. The genetically recombinant plant of Claim 1, wherein the plant expresses one or more cellulose-degrading gene products wherein the gene product or products are classified within an enzyme classification selected from the group consisting of EC 3.2.1.4 and EC 3.2.1.91.

14. The genetically recombinant plant of Claim 1, which is alfalfa or tobacco.

15. The genetically recombinant plant of Claim 14, which is alfalfa stably transformed to contain a gene sequence which encodes a cellulase-degrading enzyme selected from the group consisting of *T. fusca* cellulase E2, *T. fusca* cellulase E3, *T. ressei* CBH I, and combinations thereof.

16. The genetically recombinant alfalfa of Claim 14, which is stably transformed to contain a DNA selected from the group consisting of a coding region of SEQ. ID. NO: 1, a coding region of SEQ. ID. NO: 2, or both.

17. The genetically recombinant plant of Claim 14, which is tobacco stably transformed to contain a gene sequence which encodes a cellulase-degrading enzyme selected from the group consisting of *T. fusca* cellulase E2, *T. fusca* cellulase E3, *T. ressei* CBH I, and combinations thereof.

18. The genetically recombinant tobacco of Claim 17, which is stably transformed to contain a DNA selected from the group consisting of a coding region of SEQ. ID. NO: 1, a coding region of SEQ. ID. NO: 2, or both.

19. A method for producing cellulose-degrading enzymes comprising cultivating a genetically recombinant plant according to Claim 1.

20. The method of Claim 19, further comprising concentrating the cellulose-degrading enzymes.

21. A method for producing cellulose-degrading enzymes comprising cultivating a genetically recombinant plant according to Claim 14.

22. The method of Claim 21, further comprising concentrating the cellulose-degrading enzymes.

23. A method of ensilement comprising ensiling a plant according to Claim 1, whereby cellulose-degrading enzymes produced by the plant increase production of silage.

# TRANSGENIC PLANTS AS AN ALTERNATIVE SOURCE OF LIGNOCELLULOSIC-DEGRADING ENZYMES

## ABSTRACT OF THE DISCLOSURE

Transgenic plants which express cellulose-degrading enzymes, methods to make the transgenic plants, and methods to use the cellulose-degrading enzymes produced by the transgenic plants are disclosed.

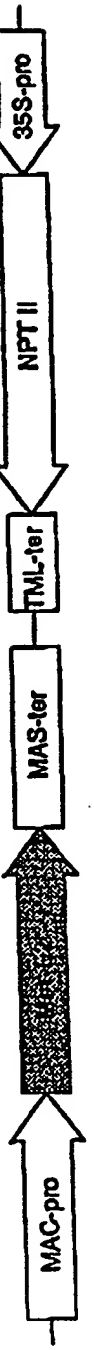

35S-pro
NPT II
TML-ter
MAS-ter
MAC-pro

FIG. 1


35S-pro
NPT II
TML-ter
MAS-ter
MAC-pro

FIG. 2

figure 3

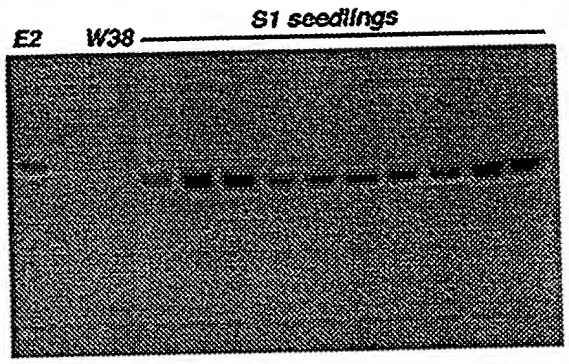



*Figure X. Expression and stability of E2 transgene in W38 tobacco.*

FIG. 3

Figure 4

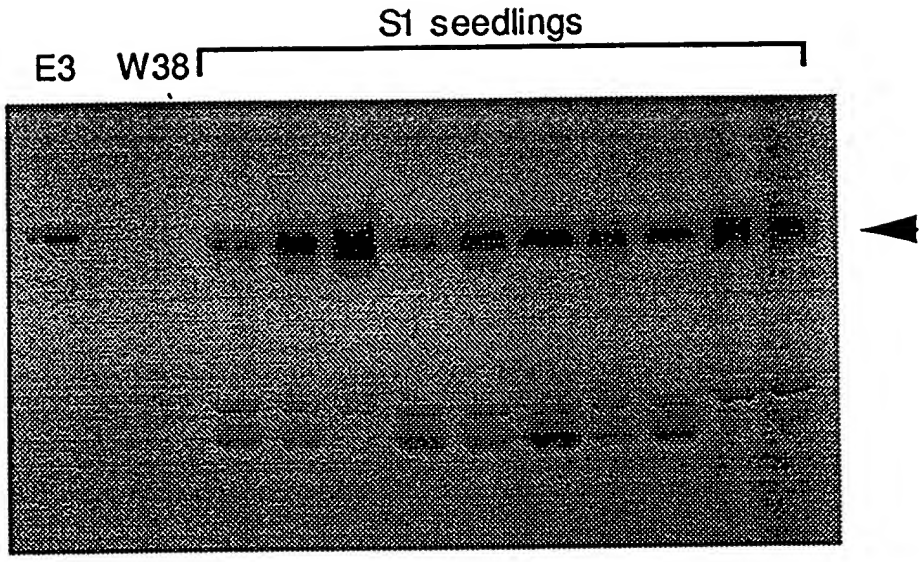



Figure 4. Expression and stability of E3 transgene in W38 tobacco.

FIG. 4

Figure 5

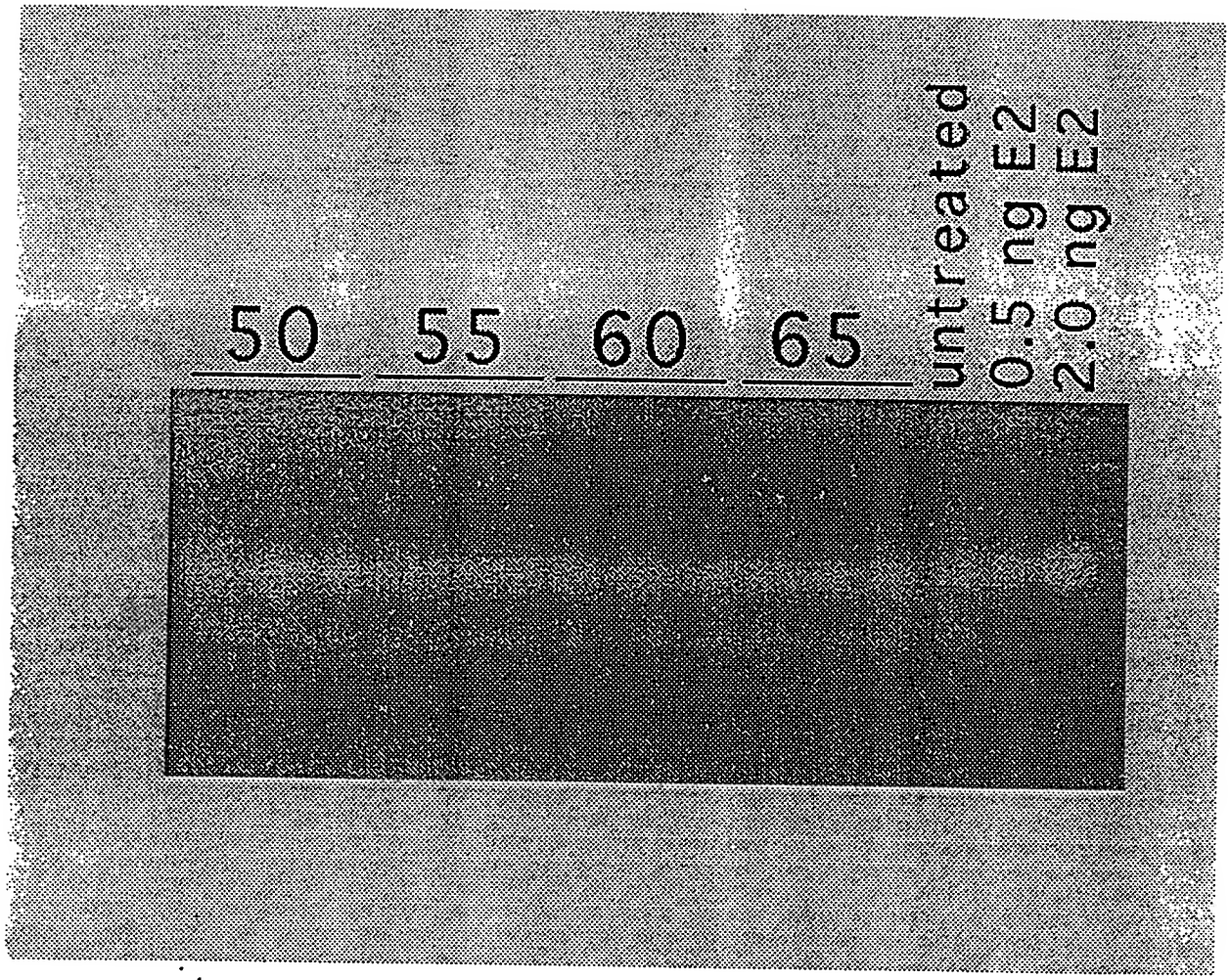



FIG. 5

# Expression of cellulases in plants...

Rationale: For an initial set of constructs, fuse E2 and E3 genes of *Thermomonospora fusca* to MAC promoter and NOS terminator in pCGN1578 constructs similar to Dennis' 321 (MnP) or 263 (LiP) but lacking any signal sequence. Based on published information, neither protein is heavily glycosylated (E3 is functional without glycosylation), so cytosolic localization should be compatible with function.

9/18/95

streaked from agar slants to LBAmp plates
D644 gave only 3 colonies (DH5α + pSZ10)
D651 " many " ( " pSZ15)

patch 2 of each for mini-preps.

1/19/96 - prep. 1/4 plate each prep using "Vince Schulz" protocol ($(NH_4)_6Ac$ ppt., CsCl ppt.)
- resuspend in 50μl, load 2μl

From maps...

E2 - 348–1578

| | |
|---|---|
| SmaI | 2 sites |
| SalI | |
| NarI | 2 sites |
| EcoRI | |
| SphI | |
| SacII | |
| BstEII | 2 sites |
| BrpMI | |
| PvuII | |

E3 - 689–2363

| | |
|---|---|
| KpnI | |
| BrpMI | " |
| BstEII | " |
| SalI | " |
| NarI | " |
| SacII | |
| NcoI | |
| BstXI | |
| SacI | " |
| HpaI | |
| SmaI | |

Thomas Fuglsang [redacted]

EXHIBIT B

# E3 construct - PCR amplification

Overall strategy:

1) generate XbaI @ 5' end, EcoRI @ 3' end (PCR)
2) subclone into pUC19 as XbaI-EcoRI fragment
3) replace ~1.4kb KpnI-SmaI of resulting pUC-E3 clone with KpnI-SmaI of pSZ10 (from strain D(44)) → sequence using universal primers
4) clone XbaI-EcoRI fragment of fully w.t. pUC-E3 into 253-1 vector (3 way ligation - XbaI-EcoRI-HindIII)
5) clone expression cassette (MAC-E3-MAS tether) into pCGN1578 as BamHI-HindIII fragment

Custom primers from UWBC (see data sheets)

XbaE3 (DSN # 5500) - designed to create an XbaI site and ATG initiation codon at the normal leader cleavage site, generating the sequence $NH_3$-MET-ALA-GLY-Cys-Ser-...
25mer of ~16225 MW, resusp. to give 210 μM solution (340 μg in 100 μl)

RIE3 (DSN # 5499) - designed to create an EcoRI site immediately after the stop codon of E3.
23mer of ~14827 MW, resusp to give 205 μM solution (307 μg in 100 μl)

set up 1st 6 of buffer optimization set

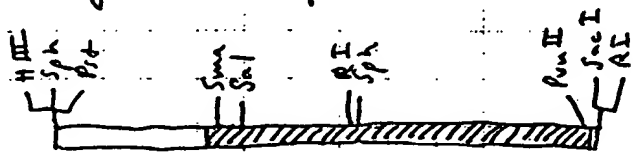
| | | combine |
|---|---|---|
| 10 μl | 10x buffer (#s 1-6) | |
| 10 μl | 4 dNTPs (2.5mM each) | |
| 1 μl | template (1/1000 dilution of pSZ10 prep) | 60 |
| 1 μl | 10 μM XbaE3 | 6 |
| 1 μl | 10 μM RIE3 | 6 |
| 77 μl | $dH_2O$ | 6 |
| 0.25 μl | Taq pol | 480 μl $dH_2O$ |

40 μl aliquots

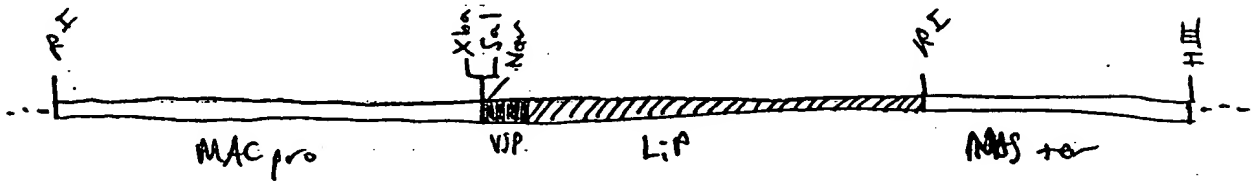
Method 1 change temp 31 to 48°C annealing temp - Thomas Foxall

# E2 cloning – strategy

E2 gene (in pUC19)

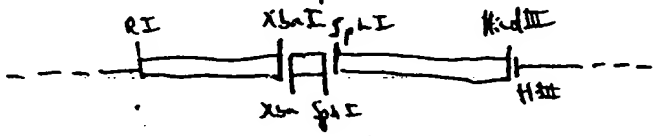


253-1 expression cassette (in pUC118, I think...)




Advantage: RI site available immediately downstream of 3' end of E2.
Problem: Need to avoid using EcoRI (2 sites in 253-1, 1 site in E2 gene)

final step in expr. cass. construction:




- vector backbone is 253-1, cut with XbaI & HindIII
- Xba-Sph fragment (~375 bp) is PCR-derived and contains the N-terminal end of E2 with new N-terminal Met (Met-Asn-Asp-Ser.... etc) This fragment will also be subcloned into pUC19 for sequencing to verify absence of errors
- Sph-Hind fragment (1.6kb) is derived from intermediate clone (~4.0kb RI fragment of E2 cloned into RI site of 253-1 [~2.5kb RI fragment excised])

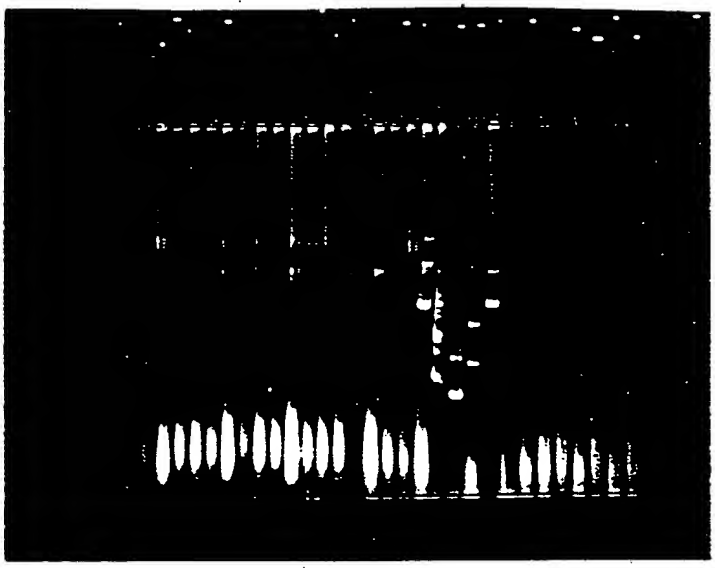
Thomas Zuglian [redacted]

# E2 & E3 - more pCGN cloning

resolve mini-preps of colonies on 0.5% gel

no plasmid! (with one exception!)

#19 is coincidentally the only isolate which grew significantly upon patching to G20 (others are reversion background)



cut 1 µl of 19 DNA
- 0.5 µl rox @ multi
- 0.25 µl SmaI
- 1 µl #19
- 7.25 µl $dH_2O$

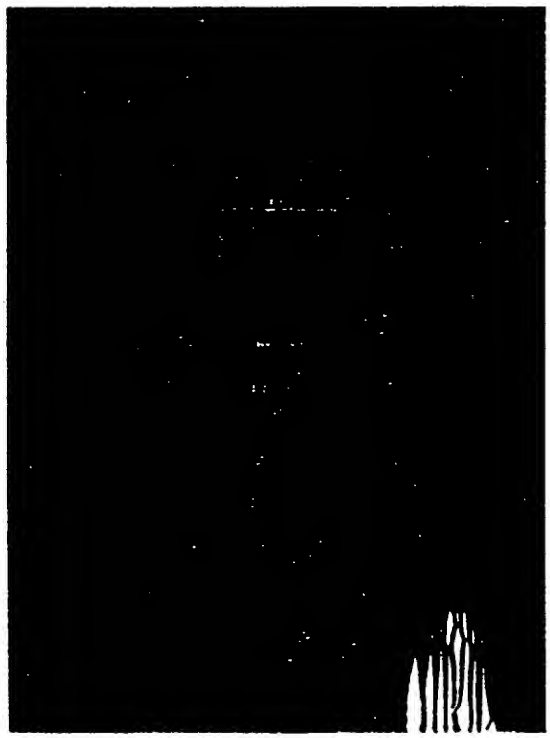
| migration distance | mouse | clinac |
|---|---|---|
| 11.3 kb | 3.6 | 3.1 |
| 7.0 kb | 3.2 | 2.7 |
| 1.3 kb | 2.4 | 1.9 |
| 2.7 kb | — | 3.3 |

see reverse for graph ...

Result:

LiP - 2.0, 2.4, 2.9, 3.2, 7.0
total = 17.5 kb

#19 - 1.4, 2.9, 3.0, 3.2, 7.0
total = 17.5 kb



Q: pCGN1577 map correct?

do more digests, but first,

transform comp LBA4404 w/ 5 µl
expell 2½ hr. @ 28°C
plate on YEP 20G @ 28°C

Thomas Zuroff

Move E2 & E3 in pCGN1578 ...

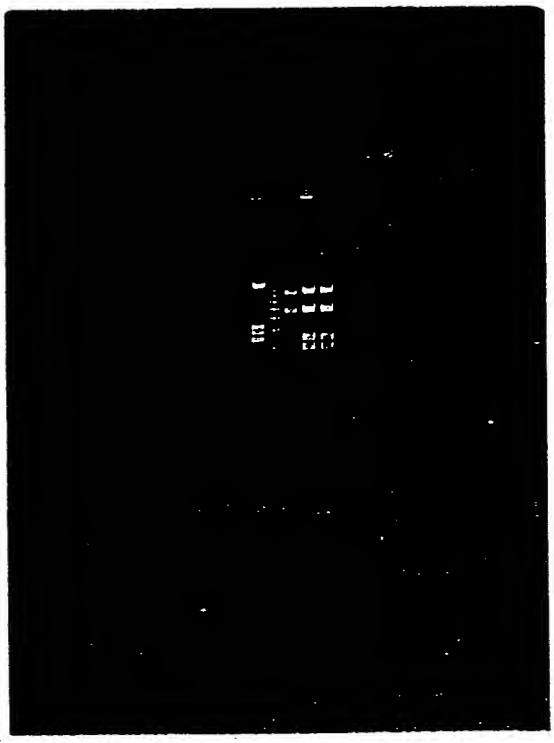
digest 318-5 with SmaI

digest 260-6, 318-5, #19 with EcoRI (mult:)

all should have 1.0, 4.6, 7.3

260, 318 should also have ~2.4 kb fragment, ~2.0 kb
E2 " " " ~0.95, 1.45, ~2.0 kb

Result: PERFECT!



318-5 - fragments of
~2.4, 2.9, 5.2, 9.5 (180 ng!)
(with SmaI)
others are as predicted

CONCLUSION:

- map of pCGN1578 is missing a SmaI site @ ~1600-1750
- E2 construct (#19) is <u>correct</u>!

THEREFORE,

- bona fide gent$^R$ transformants are recovered at very low frequency
- reversion frequency (spontaneous DH5α gent$^R$) is significant (~ $10^{-6}$-$10^{-7}$) but these are much weaker growers than gent$^R$ transformants

X [redacted]

X

# E2 & E3 - pCGN constructs

w/ig ligation mixtures described on p. 20, retransform comp DH5α and plate <u>everything</u> ...

- E2 1 ml on 3 plates
- E3 2 ml on 6 plates

Result: E2 15 lg. colonies
E3 5 " " (3 lg., 2 med., actually

Note: control transformation with 260-6 shows that lg. colonies correspond to <u>true</u> transformants (efficiency of transf. with 260-6 was comparable to pMP4.21, a pBR322 derivative)

start 1.5 ml LB 20G cultures for mini-prep (3 E2 clones, 6 E3 clones)

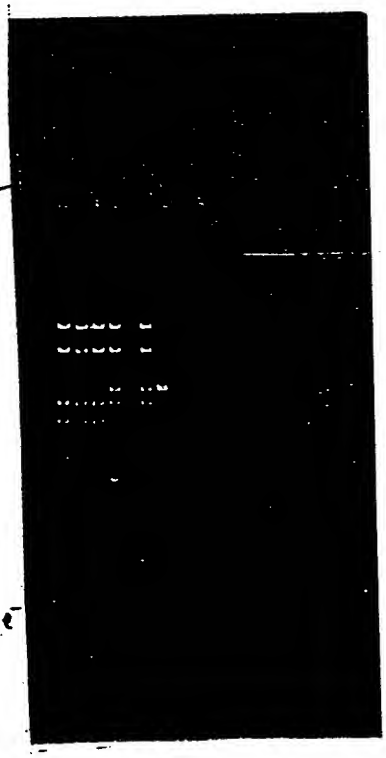
harvest after ~8 hr growth @ 37°C
E2-1-3, E3-1-3 are good E3-5,6 are weak and E3-4 is dead

3/21 - mini-prep on cell pellets

digest with EcoRI in "H" buffer

all E2 isolates look good
all E3 " " <u>bad</u>

- poor growers (on 20G) #5,6 appear to have no plasmid (as expected from previous results)
- #1,2 are indistinguishable from original 260-6 clone and probably represent background of single cut vector



# pCGN-E3, the continuing saga...

Since no "real" E3 transformants were recovered, try again

- use vector purified as previously (p20)
- use insert " " (p15)

| Ligation: | old | new |
|---|---|---|
| vector | 4 | 6.5 |
| insert | 2 | 2 |
| 10x buffer | 1 | 1 |
| T4 ligase | 0.5 | 0.5 |
| $dH_2O$ | 2.5 | — |

transformants:

test by EcoRI digestion of minilysate DNA

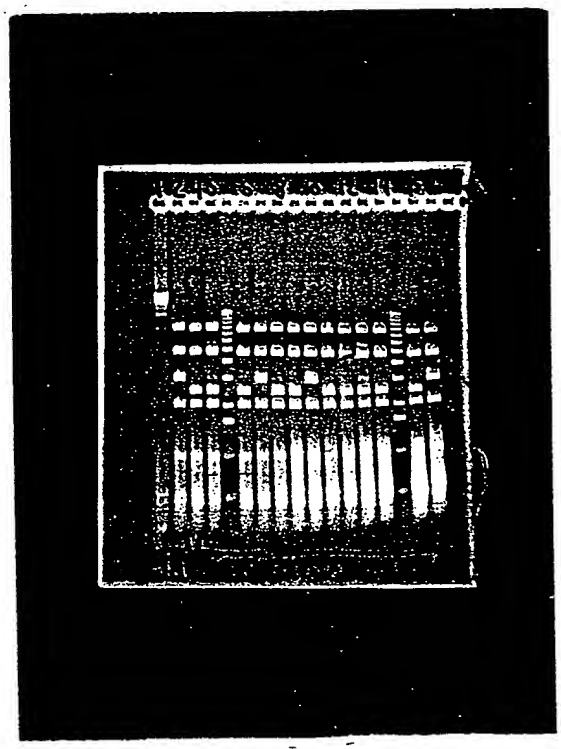
#s 2, 7, 10, 16 are correct!
with the exception of #1 (?!), all others are 260-6 recovered

transform #2 into comp tyre (2µl DNA + 100µl cells)

approx. 500 transformants obtained (YEP 20G)

start culture for plant transformation

Thomas Zügel Chaffey



# E2 - Western blot (std. dilutions, callus, leaves)

conc. of E2 is 1 mg/ml
- dilute 1:100 in 0.1 mg/ml B/t
- load 1, 2, 5, 10 µl of diluted enzyme (10, 20, 50, 100 ng)

grind tissue (Tobacco transformants) in 1x sample

use 1:50 dilution of anti E2 Ab (Diana Irwin's recommendation)

very good signal @ 10 ng E2

- approximately 20 µl of tissue (20 mg)
- assuming ~1% as soluble protein = 200 ug total
- load ~ 1/4 th of total (= 50 ng)
- signal looks like ~ 3-10 ng (i.e. - a high % of total!)

VERY PROMISING! (but is it active?...)

* plant-expressed E2 is ~ same MW as purified enzyme

# E2 – western blot

as on p27, dilute E2 stock in $dH_2O$/BSA 0.1 mg/ml

1, 2, 5, 10 µl of 1:100 dilution (10–100 ng)

test selected extracts from p29

Result: 6/3

| lane | | | |
|---|---|---|---|
| 1 | MW marker | | |
| 2 | 10 µl | E2 d.l. | |
| 3 | 5 " | " | |
| 4 | 2 " | " | |
| 5 | 1 " | " | |
| 6 | CT 1 | | none detect. |
| 7 | " 2 | | none detect. |
| 8 | " 8 | | < 1 ng |
| 9 | " 10 | | ≪ 1 ng |
| 10 | " 13 | | none detect. |
| 11 | " 25 | | ~ 2 ng |
| 12 | " 30 | | ~ 3 ng |
| 13 | " 38 | | ~ 2 ng |
| 14 | " 39 | | none detect. |
| 15 | " 30 | (1:10 dilution in ~~$dH_2O$ + BSA~~ 0.1 mg/ml) 0.2 µl | < 1 ng |

10% gel

1 µl each

Result: Western blot agrees (for the most part) with activity measurements, with some notable exceptions.

① based on CMC, CT13 should have yielded protein
② based on MUCB, CMC CT39 " " " "
③ CT25, 38 show good protein yield, despite low MUCB activity

- based on MUCB assay, expected ~ 500 ng E2
- observed less than 10 ng, therefore activity is a very poor measure of expression
- only about 0.1% total extracted protein is E2

Thomas [redacted]

# Update on plant analysis - W38/MAC-E2 (TZA1)

Analysis to date: ① reducing sugar assay using CMC as substrate, ② MUCB fluorometric assay substrate ③ Western blot using anti-E2 antiserum.

Results: ① based on Western blotting, E2 expression is @ ~ 0.1% total extracted protein for CT25, CT30, CT38 and is barely detectable in CT8, CT10. Other plants tested "contained undetectable amounts of E2". ② Although there is some correlation between CMC or MUCB activity and the presence of E2 (as determined by Western), the values obtained are orders of magnitude higher than would be expected, suggesting synergistic activity with endogenous cellulase activities. ③ Using pure E2 provided by D. Irwin, approximate Km, Vmax for MUCB are ~9 μM and ~0.12 mole/min mole, respectively.

Conclusions: A better assay method is needed. ① CMC/reducing sugar assay is probably not practical for lg. #'s of samples ② MUCB assay could be improved by (A) extracting juice directly (no buffer) followed by (B) heating @ 60°C for 30 minutes. Lower conc. of substrate (c) in the 20-100 μM range and the addition of 1 mM glucano-1,5-lactone to inhibit β-glucosidase activities may also help. Could try filter adsorbtion of heat-treated sample to further clean up (Millipore Ultrafree-DEAE MC). ③ For the time being, screen plants by Western blot until assay is improved.

Thomas Ziegelhoffer [redacted]

# E2 Western blot (W38/MAC$_p$-E2) TZA1

grind ~ 20 mg leaf tissue / in 40 µl buffer (0.1% SDS also) (50mM NaOAc pH5.5, 100 mM KCl). load 2 µl of extract / lane. save remainder for juice extraction / MUCB assay (freeze leaf t. @ -80°C)

| | | | | | |
|---|---|---|---|---|---|
| 1 | (control) | | 1 | CT85 | ✡ |
| 2 | 1 µl of E2 dilution (10 ng) | | 2 | CT86 | ✡ |
| 3 | CT6 | | 3 | CT87 | ✡ |
| 4 | CT9 | | 4 | 1 µl of E2 dilution | |
| 5 | CT11 | | 5 | CT88 | ✡ |
| ✓ 6 | CT15 | | 6 | CT89 | |
| 7 | CT16 | | 7 | CT90 | ✡ |
| ✓ 8 | CT17 | | ✓ 8 | CT91 | |
| ✓ 9 | CT18 | | 9 | CT92 | |
| 10 | CT20 | ✡ | 10 | CT94 | ✡ |
| 11 | CT22 | | 11 | CT95 | |
| ✓ 12 | CT26 | | 12 | CT96 | ✡ |
| 13 | CT36 | ✡ | ✓ 13 | CT97 | |
| 14 | CT40 | ✡ | 14 | CT99 | ✡ |
| 15 | CT84 | | 15 | CT100 | ✡ |

10% gel → transfer → Western (anti E2 solution, used 2x prev.)

RESULT: Those marked with a check show little or no E2 protein. ✡ indicates doublet (modification?) Extracts are ~ 1 mg/ml. Estimate that CT96, CT99 are ~ 2 ng phytase in 2 µg total protein.

[redacted]

# E3 western blot (tobacco transformants)

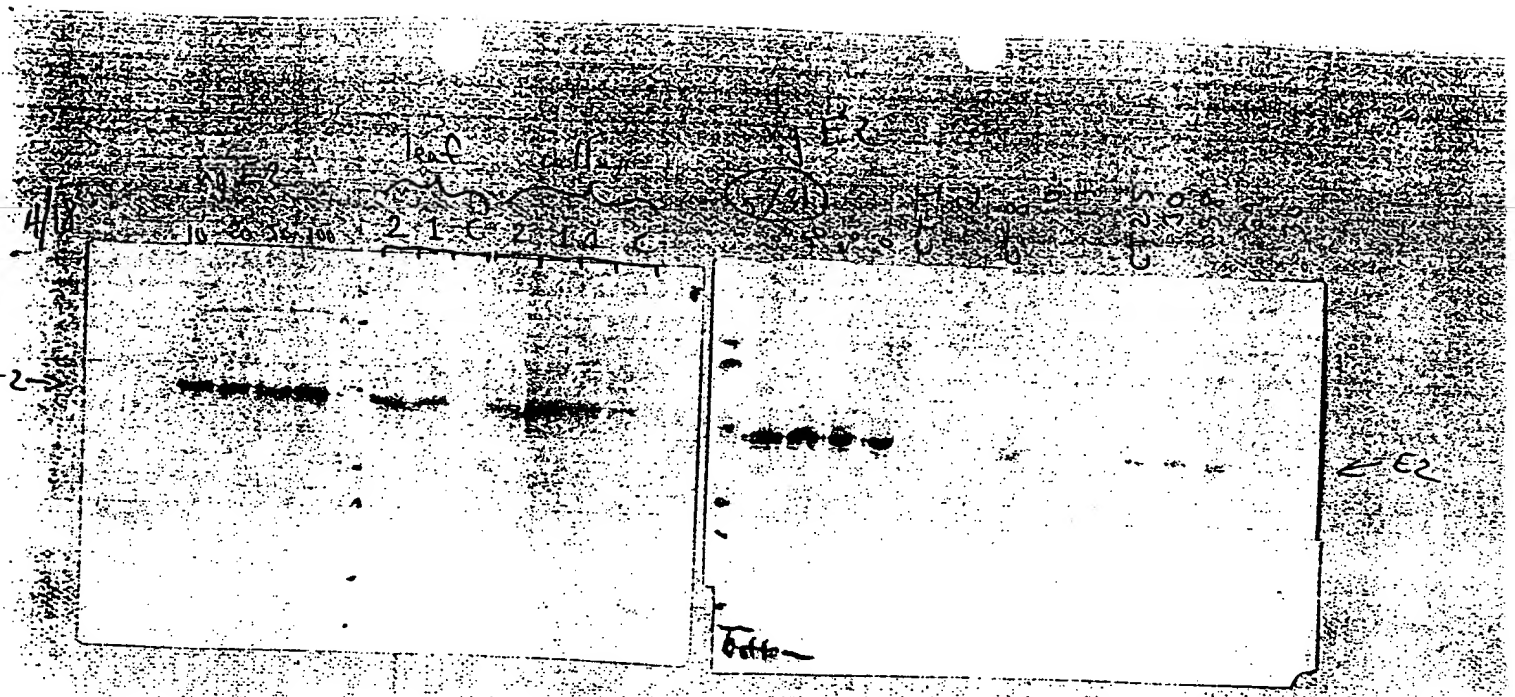
Grind ~40mg leaf tissue in 80µl "buffer D" (see p. 44), spin down pellets, recover sup (done by BK on 7/8/96).

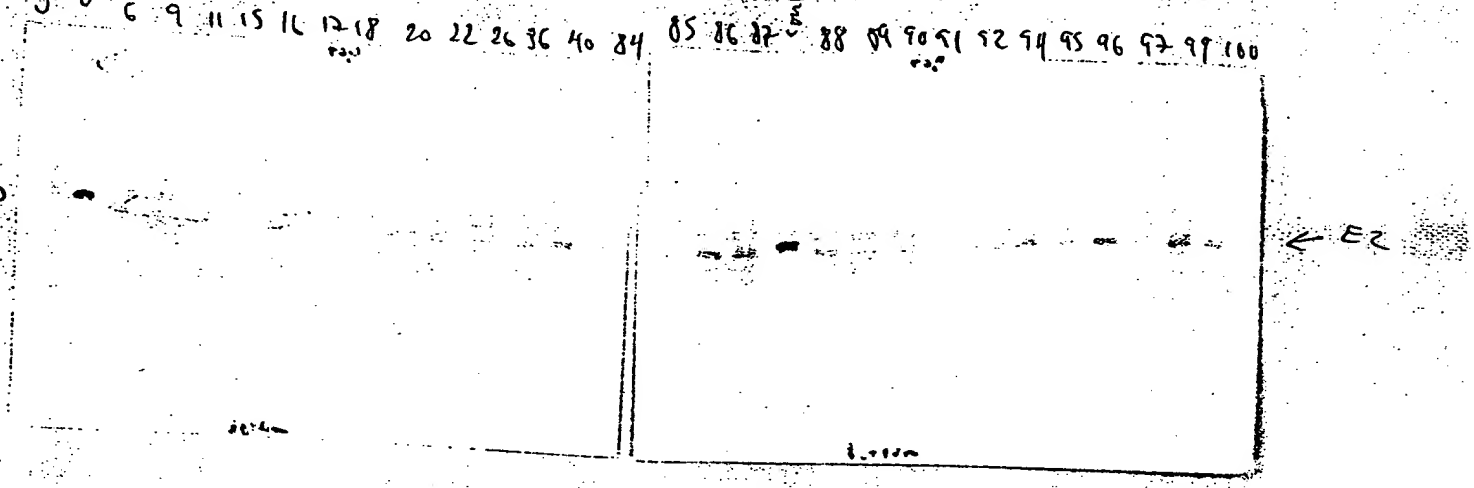
prepare samples: 20µl extract + 5µl 5x buffer

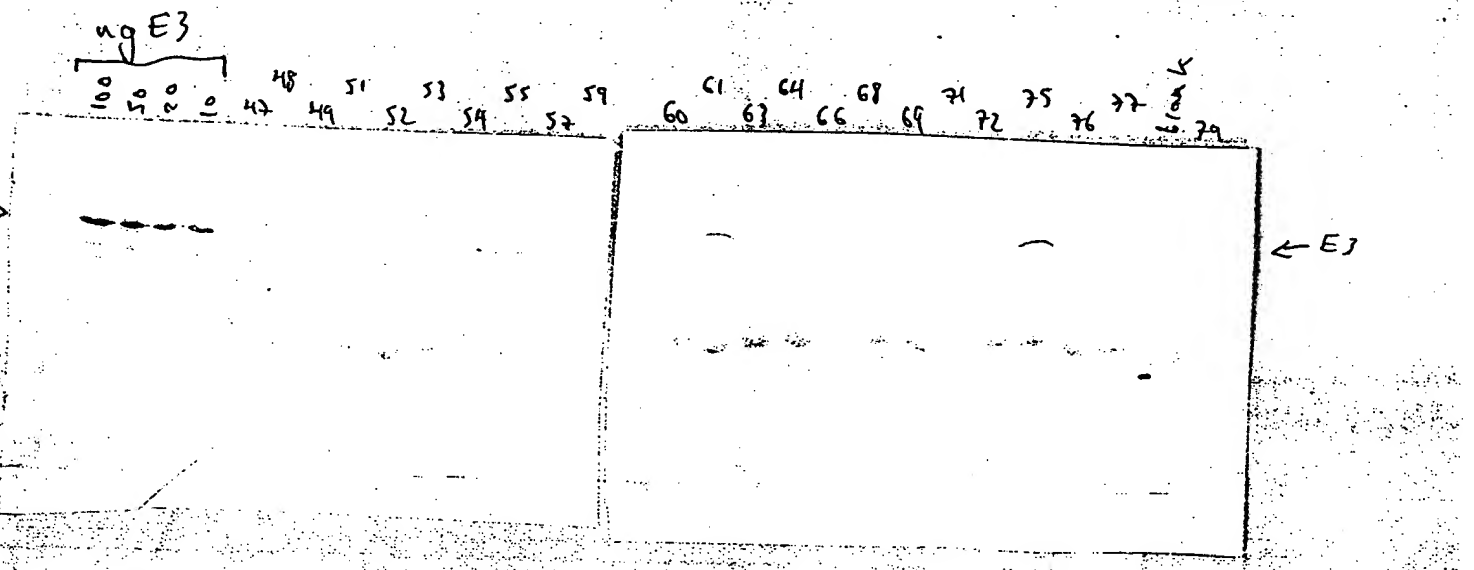
| Lane # | gel 1 | | | E3? ↓ gel 2 | | E3? | E3 diluted into dH2O + original 3SA |
|---|---|---|---|---|---|---|---|
| 1 | MW marker → | | | | | ↓ | |
| 2 | 10µl E3 1:100 | | 100ng | 8µl CT60 sample | | – | |
| 3 | 5µl " " | | 50ng | | 61 | ++ | 10% gels |
| 4 | 2µl " " | | 20ng | | 63 | +/– | |
| 5 | 1µl " " | | 10ng | | 64 | – | |
| 6 | 8µl | CT47 sample | ? (-) | | 66 | – | 1:200 of |
| 7 | " | 48 | + | | 68 | + | E3 antiserum |
| 8 | | 49 | + | | 69 | – | |
| 9 | | 51 | + | | 71 | +/– | |
| 10 | | 52 | +/– | | 72 | + | |
| 11 | | 53 | +/– | | 75 | +++ | |
| 12 | | 54 | ++ | | 76 | +/– | |
| 13 | | 55 | ++ | | 77 | +/– | |
| 14 | | 57 | ++ | | [78] | "lost" (tube leak) | |
| 15 | | 59 | – | | 79 | ++ | |

Result: all are significantly less than 10ng control
CT75 appears to be highest (maybe ≤5ng).
A cross-reacting band @ ~30 kDa was present in all lanes.

Thomas J. Close [redacted]


E2
E2


E2
E2


E3
E3

UNIVERSITY OF WISCONSIN-MADISON
Research Administration-Financial

Telephone 608-262-3822
FAX 608-262-5111

750 University Avenue
Madison, Wisconsin 53706-1490

[redacted]

Reply to Attn. of
Proposal #64511

Consortium for Plant Biotech.
Research Inc.
1220 Potter Drive
Suite 130D
West Lafayette, IN 47906

Ladies and Gentlemen:

Submitted herewith, in behalf of the Board of Regents of the University of Wisconsin System, is a revised application in the amount of $112,000 under the direction of Dr. S. Austin-Phillips, Dr. T. German and Dr. R. R. Burgess all of the Biotechnology Center.

The application for subject proposal was originally submitted under letter of [redacted]

This revised application has been administratively approved and is submitted for your consideration. Please keep our office advised as developments occur with regard to this application.

Please use the University's above-referenced proposal number in any future correspondence. Questions, or requests for further information, should be directed to Cindy Marshall at (608) 262-9727.

Sincerely,

Cheryl E. Gest

Cheryl E. Gest
Administrative Officer

CEG:CM:pf
Enclosure
cc: Asst. Dean J. Knickmeyer
Dr. S. Austin-Phillips
Dr. T. German
Dr. R. R. Burgess
Dir., Biotech. Ctr.

EXHIBIT
C

PENGAD-Bayonne, N.J.

## REVISED SCOPE OF WORK

## TRANSGENIC ALFALFA AS AN ALTERNATIVE ECONOMICAL SOURCE OF LIGNOCELLULOSIC DEGRADING ENZYMES FOR USE IN BIOMASS CONVERSION.

S. Austin-Phillips, T.L. German & R.R. Burgess.

## 1. SUMMARY.

Lignocellulosics offer tremendous potential for the production of fuel and as a chemical feedstock. This can be an economically-viable alternative to the use of fossil fuels if sugars can be efficiently and completely recovered from hydrolysis of the polysaccharide components of biomass, hemicellulose and cellulose. Acid hydrolysis of cellulose is a relatively cheap process but yields are low due to chemical alteration of sugars to non-fermentable, growth inhibitory compounds. Acid-based processes also generate a difficult and environmentally unfriendly waste stream. Enzymatic hydrolysis of cellulose gives a single, pure product, glucose, but is more costly. Enzyme production is a very large single component cost in the biomass conversion process. Thus, a major limiting factor in realizing the potential of plant biomass is the cost and availability of large amounts of cellulose-degrading enzymes. This project will determine if cellulases can be produced at a lower cost using plants as a bioreactor as compared to fermentation processes. The long term goals of the research are to develop genetically engineered alfalfa that produces high levels of enzymes (cellulases) and to develop the technology needed to extract these enzymes from alfalfa juice.

## 2. RESEARCH GOALS.

The funding for this project was reduced by 50% which will significantly impact the amount of research that can be done in a two year period. The overall research objectives and specific goals will essentially remain the same, but the actual experimentation will be scaled down.

The specifics of the research are as follows:

### A. Construct plant expression vectors carrying genes coding for cellulases.

In our original proposal we intended to obtain cellulase coding sequences from collaborators at NREL for three cellulases; (1) Cellobiohydrolase I (CBHI) from *Trichoderma reesei*, (2) a ß-1,4-endoglucanase gene originally isolated from a thermotolerant bacterium, *Acidothermus cellulolyticus* (E1), and (3) a ß-1,4-exoglucanase gene originally isolated from a different thermotolerant bacterium, *Thermomonospora fusca* (E3),(see attached letter #1). It now appears that two of these clones will not be available in the near future for our use (see letter #2). We hopefully will still be able to receive the clone for CBH-1 from NREL. However, Dr. P. Wilson from Cornell University has agreed to make available clones for two cellulases from *T. fusca*, E2 and E3, (see letter #3). Thus we will have cellulase clones for this project. Due to a reduction in funding we will probably chose one of the enzymes for our initial study instead of trying to work with all three at the same time.

Initially the cellulase coding sequences will be inserted into expression vectors we have used in the past to express foreign proteins in alfalfa. Briefly, these vectors will consist of the binary plant transformation vector pCGN1578 (McBride and Summerfelt, 1990) into which was inserted a plant expression cassette containing the coding sequence of our gene of interest. The expression cassette contains the "Mac" promoter (Comai *et al.*, 1990) and the mannopine synthase

transcription terminator (McBride and Summerfelt, 1990). Based on primary observations it may be necessary to modify the expression vectors to maximize foreign protein production and accumulation. We will remove the microbial signal sequence from the gene and determine if this results in cytoplasmic accumulation. This may be desirable since there is no substrate for the enzyme in the cytoplasm. If this does not result in cellulase expression we will use other constructs we have on hand which will allow us to compare the effect on accumulation of localization of the protein to the ER (Wandelt *et al.*, 1992), vacuole (Bednarek and Raikhel, 1991) and apoplast (Mason *et al.*, 1988). This may be important not only to maximize protein accumulation, but also to avoid any deleterious effects of the cellulases on the host plant's metabolism.

## 2. Transform alfalfa with genes coding for cellulases.

We will introduce the chimeric cellulase genes into alfalfa using procedures that are well established in our laboratory. In response to one of the reviewers comment the actual procedure is given below. This transformation method was defined after testing several modifications of the basic explant cocultivation system (Horsch *et al.*, 1985) as ways to optimize the transformation procedure. These included pretreatment of the tissue, longer cocultivation times and the use of different levels of the antibiotic kanamycin monosulphate to select for transformed tissue. The optimized procedure is as follows. New-growth trifoliates are taken from RSY27 plants maintained in a growth room (conditions as described above) and sterilized using alcohol and bleach washes (30s in 70% alcohol, 90s in 20% hypochlorite + 0.1% SDS, followed by three rinses in sterile distilled water). Leaf edges are cut on moist filter paper and tissue dropped into liquid SH-II medium (Bingham *et al.*, 1975). When sufficient explants have been taken, they are moved to a suspension of *Agrobacterium* cells (containing the engineered plasmid) from an overnight culture grown in liquid YEP selection medium. Cell density was adjusted to fall between 0.6-0.8 at $A_{660}$. After 30 minutes inoculation, the explants are gently blotted on filter paper and placed on B5H medium (Brown and Atanassov, 1985) for 4 days. They are then rinsed twice in sterile water and cultured on B5H for a further 4 days. At the end of this period, they are rinsed three times and transferred to B5H containing 25 mg $L^{-1}$ kanamycin and 250 mg $L^{-1}$ carbenicillin. Plates are maintained at 24°C, 16h photoperiod and light intensity of 60-80 $\mu E\ m^{-2}\ s^{-1}$. Explant-derived calli (and occasionally embryoids) which formed within 3 weeks on this medium are moved to B5H with antibiotics but without hormones to allow for further embryoid production and development of existing embryoids. After 3-4 weeks, embryos were transferred to MS medium (Murashige and Skoog, 1962) plus the two antibiotics to allow for development into plantlets. Callus forms on untreated explants in the presence of 25 mg $L^{-1}$ kanamycin but embryos are never produced. Each explant piece can give rise to multiple (up to 40) embryos. Plantlets are rooted on MS medium lacking antibiotics. For evaluation purposes only, one rooted plant per explant piece is assessed, thus assuring that each is the result of an independent transformation event.

The transformed plants will be analyzed for levels of enzyme production and grown to maturity to determine if there are any deleterious effects on growth and development.

We have already produced alfalfa expressing enzymes with current or potential industrial usage and are currently evaluating several methods for the efficient and economic recovery of the expressed proteins. Thus we are in an excellent position to determine if this approach is a viable alternative to fermentation for producing large amounts of enzymes needed in biomass conversion.

## 3. TIME FRAME

It is difficult to predict the time required to determine if cellulases can be expressed in plants. We estimate this will be in the range of 12 - 24 months. Maximizing expression and recovery of the enzymes will take a further 12 - 24 months.

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## 60 WORD SUMMARY

This project will determine if cellulases for use in biomass conversion can be produced in transgenic alfalfa The long term goals of the research are to develop genetically engineered alfalfa that produces high levels of cellulases and to develop the technology needed to extract these enzymes from alfalfa juice.